Acta physiol. scand. 1959. 45. 97—101.

From the Institute of Physiology, University of Helsinki, Finland.

# The Relation between the Number of Impulses and the Integrated Electric Activity in Electromyogram.

By

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Received 21 July 1958.

**Abstract.**

BERGSTRÖM, R. M. The relation between the number of impulses and the integrated electric activity in electromyogram. *Acta physiol. scand.* 1959. *45*. 97—101. — In order to evaluate electrical parameters of gross response electromyograms, the interrelationship between the integrated electric activity and the number of motor impulses of the myograms obtained from human forefinger abductor muscle in voluntary, non-isometric contractions was studied. The integrated activity was determined by planimetry. The number of motor impulses were counted irrespective of location and size. The relationship between the integrated activity and the number of motor impulses was found to be linear in contractions up to a spike frequency of 500 cps. It was concluded that, as far as small muscles are concerned, counting of the motor impulses in the electromyogram, recorded with non-selective electrodes, can be used in estimating the electric activity of the whole muscle.

In studies designed to elucidate the correlations between the mechanical and electrical activity of striated muscles, attention has been paid, in appraising electromyograms, to the number of

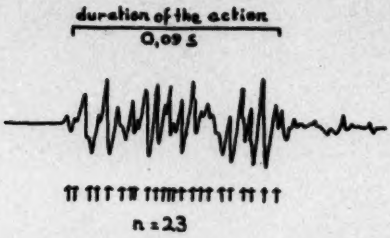


Fig. 1. The counting of the number of the motor spikes in electromyogram, recorded with non-selective electrodes. $n$ = number of action potentials. Arrows indicate the "spikes" counted.

action potentials and to their electric voltage. In so far as research dealing with whole muscles and using the non-selective electrode technique is concerned and the records accordingly contain potential variations of several motor units, certain defects attach to both of the parameters yielded by the electromyograms.

As the potential frequency increases, the potentials arising in the different parts of the muscle overlap and the counting of impulses is rendered more difficult. Similarly, summation and subtraction between potentials happens in the electromyogram, with the result that the value obtained for the electric activity of the muscle on the basis of the area of the myogram does not agree with the true value. It has been assumed, however, that the proportion of overlap between potentials remains constant when the electric activity increases (BIGLAND and LIPPOLD 1954).

Up to the potential frequency where the majority of the myogram impulses can still appear as completely separate (200 cps approximately, if account is taken of the duration of the potential of a motor unit, about 5 msec, BUCHTHAL, GULD and ROSENFALCK 1954) the change in the area of the electromyogram corresponds to that of the number of potential spikes, provided that synchronization is slight. If BIGLAND and LIPPOLD's assumption holds true, it may nevertheless be supposed that this proportionality remains valid even when the frequncy of the muscle action currents exceeds the said value, since the "spikes" remain, notwithstanding the partial overlap of potentials, separate even at higher frequencies (the action potential having the form of a "spike potential").

According to the foregoing it is of interest to compare the

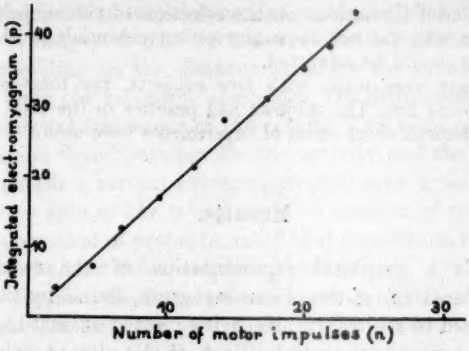


Fig. 2. The relation between the number of motor impulses and the area of electro-myogram. Recording by means of surface electrodes from m. interosseus dors. I in voluntary, non-isometric action. Integrated electromyogram area in arbitrary units ($p$). Number of impulses in electromyogram ($n$).

number of impulses in an electromyogram with the integrated electric activity of the muscle as estimated from the area of the electromyogram.

## Methods.

The experimental subjects were required to perform voluntary actions by the forefinger abductor (m. interosseus dors. I), abduction varying from 0 to 30°. The mechanical impulse (k.dt, k = force and dt = time) produced by the muscle in action, estimated at the distal insertion point, varied between 0 and $10^5$ cgs units (for the method used in estimation, see BERGSTRÖM 1957).

Action potential recordings were made from the muscle using surface electrodes (Ø 0.3 cm on the muscle and 0.6 cm on the distal-dorsal part of the forearm), an asymmetric amplifier and an oscillograph. The film used in photographing travelled at 50 cm/sec. The positions of the electrodes as well as the degree of amplification were kept constant. To estimate the electric activity the area of the electromyogram was determined by planimetry (see LIPPOLD 1952). In counting the motor impulses of the electromyogram we did not adopt, unlike in a previous study (BERGSTRÖM 1958), the method where the "disturbance region" in the immediate neighbourhood of the isoelectric line is disregarded (JALAVISTO *et al.* 1938). In the present study the impulses were counted regarding every "spike", irrespective of its location, as an action potential (Fig. 1), so that no disturbance region was excluded. This

procedure placed limitations on the selection of the subjects in that only persons who did not show any action potentials in the muscle, when in rest, could be admitted.

Experiments were made with five subjects, the total number of recordings being 250. The subjects had practice in the task. To avoid fatigue phenomena short series of experiments were used.

## Results.

Fig. 2 is a graphical representation of the results as a whole, the area ($p$) of the electromyogram, found by planimetry and expressed in arbitrary units, being plotted against the number ($n$) of the myogram potentials. Each of the plotted points is the mean for 25 observations. The standard deviation varies between $\pm$ 0.02 and $\pm$ 1.12 in the ordinate and between $\pm$ 0.04 and $\pm$ 1.09 in the abscissae direction. No essential differences between the results for the different subjects could be discovered.

The highest frequency measured from the electromyogram (an estimated average for 0.01 sec) was 500 cps. The mechanical impulse at the insertion point of m. interosseus dors. I was then $0.78 \cdot 10^5$ cgs units.

It is evident from the figure that the number of motor impulses changes in direct proportion to the electrical activity of the electromyogram.

## Discussion.

In comparing the integrated activity of the electromyogram, recorded by means of surface electrodes from m. interosseus dors. I during abduction of the forefinger, to the number of motor impulses it was found, in the experimental conditions described, that the relation between them was linear. This result is at variance with the result we obtained in a previous study (BERGSTRÖM 1958), in which impulses situated in a "disturbance region" were excluded. In the experiments reported here all electromyogram "spikes" were taken into account.

The linear relation found can be assumed to apply to muscle activity where no synchronization of the action currents is present. In the study reported here the series of experiments performed in each sitting were short so that fatigue and the synchronization phenomena which may result from fatigue were avoided.

From the result obtained it can be concluded that even though the size (voltage) of the recorded electromyogram potentials varies, depending on the distance at which the motor unit concerned is from the recording electrodes, the differences disappear in the statistical treatment of the results. This is to say that the linear relation found between electric activity and the number of impulses entails a certain average potential area $p_o$ so that $p_o = a \cdot p/n$ ($p$ = area of the myogram, $n$ = number of motor spikes and $a$ = a constant of proportionality that depends on the position and size of the electrodes, amplification etc.).

If, as is assumed by BIGLAND and LIPPOLD (1954), the addition and subtraction between potentials in the electromyogram remains constant when the electric activity of the muscle increases, the result obtained means that, at least as far as small muscles are concerned, the counting of the motor impulses in the electromyogram, recorded with non-selective electrodes, can be used in estimating the electric activity of the whole muscle. The presented linearity was still valid at the action potential frequency of 500 cps.

## Summary.

In the reported experiments it was possible to demonstrate, in electromyograms recorded by surface electrodes from m. interosseus dors. I in connection with voluntary, non-isometric action, a linear interrelation of the integrated area of the electromyogram and the number of action potentials.

I wish to express my thanks to the Finnish State Committee for Natural Sciences for a grant which has made it possible to carry out the work reported here.

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Acta physiol. scand. 1959. 45. 102—108.

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# Studies on the Effect of Other Alcohols on the Metabolism of Methanol in Rat Liver Homogenates.

By

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Received 28 August 1958.

**Abstract.**

KOIVUSALO, M. Studies on the effect of other alcohols on the metabolism of methanol in rat liver homogenates. *Acta physiol. scand.* 1959. *45*. 102—108. — The effect of various lower aliphatic alcohols on the accumulation of formaldehyde in rat liver homogenates incubated with methanol has been studied. The addition of ethanol inhibited very clearly the accumulation of formaldehyde, n-propanol, iso-propanol or n-butanol had no effect on the accumulation of formaldehyde.

Ethanol has been repeatedly shown to retard the utilisation of methanol in the animal organism. This effect of ethanol has a considerable toxicological importance apart from its theoretical implications, since most persons who drink methanol consume it with ethanol as in the various denaturated spirits. It has been shown in clinical studies that methanol poisoning is less severe when ethanol also has been consumed, and ethanol has been used in the therapy of methanol poisoning (RØE 1943, 1946). The effect of ethanol is seen also in rat and guinea pig liver slices and homogenates (KOIVUSALO 1956).

In this paper results of experiments are presented in which the effect of higher aliphatic alcohols on the metabolism of methanol has been studied *in vitro*. Ethanol, *n*-propanol, *iso*-propanol, and *n*-butanol have been incubated in addition to methanol with rat liver homogenates, and the accumulation of formaldehyde has been determined.

## Methods.

White female rats of Wistar strain weighing 260—290 g were used as experimental animals. They were fed the usual stock diet of this Department.

The animals were not fasted before the experiments. They were killed with a blow on the head, their livers were rapidly removed, cooled on ice and homogenised with a Bühler homogeniser to make a 10 per cent homogenate. 0.1 M potassium phosphate buffer pH 7.4 was used as homogenising medium. The incubations were carried out in a temperature-controlled water bath with a constant-shaking device at 37° C. The gas phase was air in all experiments. Samples of 1 ml were taken from the incubation flasks before and after the incubation and in some experiments also during the incubation. The samples were transferred into centrifuge tubes containing 5 ml of 10 per cent trichloroacetic acid for deproteinisation. The clear supernatant obtained after centrifugation was used for the determinations.

The determinations of formaldehyde and methanol were made using the chromotropic acid reaction, modified as described earlier (Koivusalo 1956).

## Results.

The results of typical experimental series are presented in Fig. 1 and in Table I.

From the curves in Fig. 1 it is seen that ethanol has a very clear inhibitory effect on the accumulation of formaldehyde in rat liver homogenates incubated with methanol. On the other hand *n*-propanol and *n*-butanol had no effect on the accumulation of formaldehyde.

Similar results were obtained although the amounts of added alcohols were increased up to 600 $\mu$M (Table I). Also the addition of *iso*-propanol was without effect on the accumulation of formaldehyde.

The utilisation of methanol was also determined in some experimental series. It was found to be directly proportionate to the accumulation of formaldehyde as has been found also earlier (Koivusalo 1956).

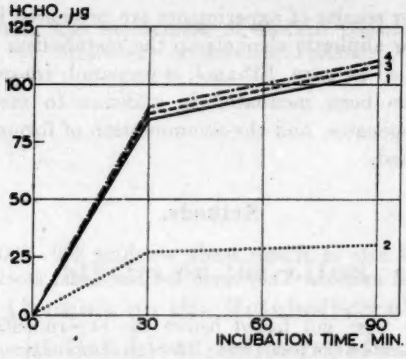


Fig 1. Effect of other alcohols on the accumulation of formaldehyde in rat liver homogenate incubated with methanol.

5 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 800 µg of methanol. Total volume 7 ml. Incubated at 37°.

Curve 1. No additions.
Curve 2. 200 µM of ethanol.
Curve 3. 200 µM of *n*-propanol.
Curve 4. 200 µM of *n*-butanol.

**Table I.**

*Effect of other alcohols on the accumulation of formaldehyde in rat liver homogenates incubated with methanol.*

Experimental conditions as in Fig. 1. Incubated for 60 minutes at 37°. Same homogenate was used in all experiments.

| | Formaldehyde accumulated µg |
|---|---|
| No additions | 102 |
| 300 µM ethanol | 25 |
| 600 µM ethanol | 15 |
| 300 µM *n*-propanol | 97 |
| 600 µM *n*-propanol | 86 |
| 300 µM *iso*-propanol | 97 |
| 600 µM *iso*-propanol | 101 |
| 300 µM *n*-butanol | 108 |
| 600 µM *n*-butanol | 103 |

## Discussion.

The present experiments demonstrate very clearly that at least under these experimental conditions only ethanol inhibits the accumulation of formaldehyde and presumably also the oxidation of methanol in rat liver homogenates. *N*-propanol, *n*-butanol and *iso*-butanol had no effect although their concentration was increased up to 24 times the concentration of methanol.

Ethanol has been widely used as a therapeutic agent in the methanol poisoning, after RØE (1942, 1946) had shown in clinical studies that the poisoning caused by methanol was less severe when ethanol was also ingested. The increased formic acid excretion in the urine which is found after ingestion of methanol is diminished by simultaneous administration of ethanol in dogs and rabbits (ASSAR 1914, BASTRUP 1947) as well as in man (KENDAL and RAMANATHAN 1953). Ethanol increases also the elimination of unchanged methanol in the urine (LEAF and ZATMAN 1952). The oxidation of isotope-labelled methanol to carbon dioxide in rats *in vivo* is strongly inhibited by the simultaneous administration of ethanol (BARTLETT 1950). Ethanol also retards the elimination of methanol from blood in rabbits (AGNER and BELFRAGE 1946, KOIVUSALO 1956).

Oxidation of methanol by crude alcohol dehydrogenase from horse liver prepared according to LUTWAK-MANN (1938) is inhibited by ethanol, as may be judged from the decreased amount of formaldehyde formed (ZATMAN 1946). Added ethanol likewise depressed the oxidation of isotope-labelled methanol by rat liver slices as a linear function of the logarithm of the ethanol concentration (BARTLETT 1950). In experiments with rat and guinea pig liver homogenates incubated with methanol, the addition of ethanol caused a clear inhibition both in the utilisation of methanol and in the accumulation of formaldehyde (KOIVUSALO 1956). When the values for the utilisation of methanol in these experiments were plotted according to LINEWEAVER and BURK (1934) it could be concluded that the inhibition caused by ethanol was most likely of a competitive nature.

Thus we have convincing evidence of the inhibitory effect of ethanol on the metabolism of methanol. The most natural explanation of this inhibition would be that methanol and ethanol are oxidised by the same enzyme, which has greater affinity for ethanol than methanol, which causes the competitive inhibition by

ethanol. However, the enzymic mechanism responsible for the oxidation of methanol *in vivo* is not yet surely known. The liver alcohol dehydrogenase, crystallised by BONNICHSEN and WASSÉN from horse liver, does not react with methanol and diphosphopyridine nucleotide to any extent according to THEORELL and BONNICHSEN (1951), a fact which has been confirmed also in this laboratory. However, more crude preparations of horse liver alcohol dehydrogenase are able to reduce diphosphopyridine nucleotide in the presence of methanol, although the rate is much slower than with ethanol (KOIVUSALO 1956). As a possible explanation for the failure of crystalline liver alcohol dehydrogenase to oxidise methanol, THEORELL and BONNICHSEN (1951) have suggested that the oxidation of methanol possibly needs also some other co-factor than diphosphopyridine nucleotide alone.

Catalase can also oxidise alcohols via a coupled oxidation system, which consist of a primary oxidising system generating hydrogen peroxide and of alcohol and catalase, which latter functions peroxidatically in these reactions (KEILIN and HARTREE 1936, 1945). The primary oxidising system is formed by oxidases which catalyse the oxidation of their substrates by molecular oxygen, reducing the latter to hydrogen peroxide. CHANCE (1947) has calculated that liver catalase content is sufficient to account for the rate of methanol disappearance *in vivo*, and it has been suggested (JACOBSEN 1952) that all the methanol and one-fifth of the ethanol metabolised in the organism were oxidised by catalase. However, the very effective inhibition by ethanol is not very likely in this system, because catalase oxidises methanol and ethanol at about the same rate. There is, however, also the possibility that ethanol inhibits the primary oxidising systems.

When the oxidation of other lower aliphatic alcohols is considered, it is seen that we have little actual knowledge of their metabolic fate in the animal organism. The horse liver preparation of LUTWAK-MANN (1938) oxidised also *n*-propanol and amyl alcohol at medium rates between those of ethanol and methanol. The crystalline horse liver alcohol dehydrogenase reacted according to THEORELL and BONNICHSEN (1951) with higher homologues of ethanol if they contained the group $= C—CH_2OH$, thus for instance *iso*-propanol does not react, but *n*-propanol and *n*-butanol will react with the pure horse liver enzyme. In experiments on the initial reaction velocity they found that the Michaelis constants were lower for allyl-alcohol, *n*-propanol and *n*-butanol than for eth-

anol, but the maximal reaction velocities were the same for all as was also required by the theory they presented for the action of liver alcohol dehydrogenase. In the coupled oxidation systems of KEILIN and HARTREE (1945) methanol and ethanol were oxidised at almost the same rate, *n*-propanol was also oxidised although more slowly, and *iso*-propanol, *n*-butanol and amyl alcohol very slowly if at all.

When the failure of other alcohols than ethanol to inhibit the oxidation of methanol to formaldehyde, which was constantly observed in the present study, is taken into consideration, certain difficulties are found in explaining this observation. If alcohol dehydrogenase with possibly some auxiliary system should be responsible for the oxidation of methanol, *n*-propanol and *n*-butanol should also have had inhibitory effects. Oxidation of methanol by the catalase system is more readily reconciled with these results, because other alcohols are oxidised by it much more slowly than ethanol and methanol. However, some inhibition by *n*-propanol might have been expected also in this system. *Iso*-propanol is oxidised neither in the alcohol dehydrogenase nor in the catalase system, so its failure to inhibit the formation of formaldehyde is very comprehensible.

AEBI, KOBLET and VON WARTBURG (1957) have quite recently reported results from studies on the biological oxidation of methanol, in which also the effect of homologous alcohols was studied, both *in vivo* and *in vitro* in liver slices. The amount of radioactive carbon dioxide formed from $C^{14}$-methanol was used as the measure of the oxidation of methanol. They found that in these systems *n*-propanol on a molar basis inhibited the oxidation of methanol as much as or more than ethanol, and some inhibition was effected also by *n*-butanol. The discrepancy between these results and those obtained in the present study may be due to the different experimental techniques, by which the formed formaldehyde on the one hand and oxidation to carbon dioxide on the other were determined. It is also possible that the oxidation of methanol has different pathways *in vivo* and also in different tissue preparations. The ratios between the participation of catalase and alcohol dehydrogenase systems in the oxidation of methanol may for instance differ according to experimental conditions.

## Summary.

1. The effect of various lower aliphatic alcohols on the accumulation of formaldehyde in rat liver homogenates incubated with methanol has been studied.

2. The addition of ethanol inhibited very clearly the accumulation of formaldehyde, *n*-propanol, *iso*-propanol or *n*-butanol had no effect on the accumulation of formaldehyde.

3. The results are discussed in the light of the different theories on the mechanism of methanol oxidation in the animal organism.

This work has been aided by a generous grant from the Foundation for the Research of Alcohol Problems.

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Acta physiol. scand. 1959. *45*. 109—115.

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# Studies on the Metabolism of Methanol and Formaldehyde in vitro in Molybdenum Deficiency Induced by Tungstate Feeding.

By

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Received 28 August 1958.

**Abstract.**

KOIVUSALO, M. Studies on the metabolism of methanol and formaldehyde in vitro in molybdenum deficiency induced by tungstate feeding. *Acta physiol. scand.* 1959. *45*. 109—115. — The formation of formaldehyde from methanol in liver homogenates was almost totally abolished in molybdenum-deficiency induced by tungstate feeding. The addition of diphosphopyridine nucleotide, adenosine triphosphate or xanthine was not able to increase the formation of formaldehyde from methanol in the molybdenum-deficient liver homogenates as it did in normal homogenates. The utilisation of added formaldehyde was also depressed in the molybdenum-deficient homogenates. The liver xanthine oxidase activity fell to very low values after the feeding of tungstate, but the catalase activity of liver was not affected by it.

Formaldehyde can be oxidised *in vitro* by several liver enzymes, namely by diphosphopyridine nucleotide dependent aldehyde dehydrogenase (RACKER 1948), xanthine oxidase (MORGAN 1926), aldehyde oxidase (CARPENTER 1951), glyceraldehyde-3-phosphate dehydrogenase (NYGAARD and SUMNER 1952), catalase (CHANCE

**Table I.**

*Effect of tungstate feeding on the accumulation of formaldehyde in undialysed rat liver homogenates incubated with methanol.*

5 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 800 µg of methanol. Incubated at 37° for 60 minutes.

| Diet | Formaldehyde accumulated µg | | |
|---|---|---|---|
| | Exp. A. | Exp. B. | Exp. C. |
| Control ..... | 70 | 62 | 53 |
| W + Mo ..... | 62 | 56 | 57 |
| W ..... | 14 | 8 | 7 |

1951), and by a specific diphosphopyridine nucleotide-glutathione dependent formaldehyde dehydrogenase (STRITTMATTER and BALL 1955). Of these enzymes, xanthine oxidase and aldehyde oxidase are both metalloflavoproteins containing molybdenum (MACKLER, MAHLER and GREEN 1954, MAHLER *et al.* 1954). These two enzymes are also linked to the oxidation of methanol to formaldehyde if this is assumed to take place via a coupled oxidation catalysed by catalase functioning peroxidatically (KEILIN and HARTREE 1936, 1945). Xanthine oxidase and aldehyde oxidase can serve here with their respective substrates as a primary oxidising system generating hydrogen peroxide.

Rat tissues can be depleted of molybdenum by feeding them a diet containing tungstate (HIGGINS, RICHERT and WESTERFELD 1956). The xanthine oxidase activities of the tissues are also very low in this molybdenum deficiency.

This study was designed to discover how far the molybdenum containing enzymes are involved in the metabolic reactions of methanol and formaldehyde in rat liver homogenates. Also the catalase and xanthine oxidase activities of the used liver homogenates were determined.

## Methods.

White female rats of Wistar strain, weighing 200—250 g were used as experimental animals. They were divided into three groups. The rats in the first group received daily in addition to the usual stock diet

**Table II.**

*Effect of tungstate feeding on the accumulation of formaldehyde in dialysed rat liver homogenates incubated with methanol.*

Experimental conditions as in Table I. Homogenate was dialysed against 0.1 M potassium phosphate buffer for 24 hours. Additions: 2.5 μM DPN; 5 μM ATP; 5 μM xanthine.

| Diet | Additions | Formaldehyde accumulated μg | | |
|---|---|---|---|---|
| | | Exp. A. | Exp. B. | Exp. C. |
| Control | None ..... | 30 | 20 | 16 |
| » | DPN ..... | 52 | 46 | 47 |
| » | ATP ..... | 80 | 83 | 89 |
| » | Xanthine ..... | 71 | 68 | 76 |
| W + Mo | None ..... | 25 | 20 | 15 |
| » | DPN ..... | 52 | 50 | 50 |
| » | ATP ..... | 85 | 95 | 101 |
| » | Xanthine ..... | 70 | — | 74 |
| W | None ..... | 18 | 7 | 10 |
| » | DPN ..... | 18 | 0 | 8 |
| » | ATP ..... | 20 | 3 | 15 |
| » | Xanthine ..... | 16 | 4 | 13 |

of this Department 7.5 mg of $Na_2WO_4$ each, and in the second group 7.5 mg of $Na_2WO_4$ and 5.0 mg of $Na_2MoO_4 \cdot 2\ H_2O$ each. The third group served as controls without any additions to the diet. The experimental series A was carried out after feeding on the above diets for 2 months, series B after 3 months and C after 4 months. Two rats from each group were included in an experimental series.

The liver homogenates were prepared as described earlier (KOIVUSALO 1956, 1958). The determinations of formaldehyde and methanol were made by methods based on the chromotropic acid reaction (KOIVUSALO 1956). All the values in the tables are averages from duplicate determinations from two similar incubation flasks and they are corrected for the tissue and reagent blank values.

The catalase determinations were made by the perborate method of FEINSTEIN (1949), using dilutions of liver tissue 1 : 1,000 and 1 : 500. The results given are averages from these determinations.

The xanthine oxidase activity was determined using the manometric method of WESTERFELD and RICHERT (1952). In the experimental series A and B approximate determinations were made with a method based on the reduction of triphenyl tetrazolium. The method was similar to that described by MITIDIERI *et al.* (1955).

**Table III.**

*Effect of tungstate feeding on the utilisation of formaldehyde in rat liver homogenates.*

Experimental conditions as in Table I, except substrate 500 μg formaldehyde. The values are corrected for blanks in which boiled homogenate has been used.

| Diet | Formaldehyde utilised μg | |
|---|---|---|
| | Exp. B. | Exp. C. |
| Controls ..... | 175 | 205 |
| W + Mo ..... | 189 | 190 |
| W ..... | 129 | 110 |

## Results.

No gross toxic effects were seen in the rats after the administration of tungstate either alone or with molybdate.

The results of the experiments are presented in Tables I—IV.

The feeding of tungstate inhibited very clearly the accumulation of formaldehyde in liver homogenates incubated with methanol (Tables I and II). No inhibition was seen when molybdate was also administered, the results were then approximately the same as those obtained with normal rats. The addition of diphosphopyridine nucleotide, adenosine triphosphate and xanthine into the dialysed homogenates did not increase the formation of formaldehyde as it did in both the control groups. The results obtained after feeding the experimental diets for 2, 3 or 4 months did not appreciably differ.

The utilisation of methanol was also determined in some of the experiments and it was found to be very low in the liver homogenates from the tungstate fed rats, which was also indicated by the diminished accumulation of formaldehyde. The utilisation of added formaldehyde in the liver homogenates was diminished after tungstate feeding, as is seen from the results presented in Table III.

The liver catalase content was not affected by the molybdenum deficiency but the liver xanthine oxidase activity fell to very low values after tungstate feeding, as is seen from the results presented in Table IV. In the first two experimental series A and B the liver xanthine dehydrogenase activities were only approximately de-

**Table IV.**

*Effect of tungstate feeding on the liver catalase and xanthine oxidase activities.*

Experimental series C. Catalase assay and units according to FEINSTEIN (1949), xanthine oxidase assay and units according to WESTERFELD and RICHERT (1952).

| | Controls | W | W + Mo |
|---|---|---|---|
| Liver catalase ..... | 0.70 | 0.75 | 0.68 |
| Liver xanthine oxidase ... | 28 | 5 | 24 |

termined by a colorimetric method based on the reduction of triphenyl tetrazolium. The results obtained showed also very low values after tungstate feeding.

## Discussion.

The results of the experiments presented in this paper clearly demonstrate that the metabolism of methanol in liver homogenates is grossly impaired in molybdenum deficiency induced by tungstate feeding. No effects were seen when molybdenum was also given to the rats, which indicates that the effects were in fact due to the depletion of molybdenum from the tissues.

The effects of dialysis and addition of diphosphopyridine nucleotide, adenosine triphosphate and xanthine are in accordance with the earlier studies using rat liver homogenates (KOIVUSALO 1956, 1958). The failure of these co-factors to stimulate the formation of formaldehyde from methanol in homogenates from molybdenum-deficient livers is further indication that their effect in normal homogenates may be due to the fact that they can serve as substrates for xanthine oxidase.

The utilisation of formaldehyde in rat liver homogenates seems to be rather independent of enzymes containing molybdenum, as is seen from the relatively slight decrease in the utilisation of formaldehyde in molybdenum-deficient liver homogenates.

The liver catalase content was not affected by the feeding of either tungstate or tungstate together with molybdate. The inhibition found in the formation of formaldehyde from methanol in molybdenum-deficient liver homogenates thus could not be due to lack of catalase.

The liver xanthine oxidase activity fell to a very low level after the feeding of tungstate but was within normal limits when molybdate was also administered. This is well in accordance with the earlier studies of HIGGINS, RICHERT and WESTERFELD (1956). The slight residual xanthine oxidase activity found in the present experiments is probably due to the fact that adult animals were used and no attempt was made to remove molybdenum from the stock diet.

The results of the present experiments may be taken as evidence for the participation of xanthine oxidase and possibly also other molybdenum dependent enzymes in the oxidation of methanol. These results are readily explained if the oxidation of methanol to formaldehyde in rat liver homogenates is assumed to take place via a coupled oxidation system catalysed by catalase and xanthine oxidase (KEILIN and HARTREE 1946).

## Summary.

1. The formation of formaldehyde from methanol has been studied in homogenates prepared from livers of normal and molybdenum-deficient rats. The molybdenum-deficiency was induced by tungstate feeding.

2. The formation of formaldehyde from methanol in liver homogenates was almost totally abolished in the molybdenum-deficiency. The addition of diphosphopyridine nucleotide, adenosine triphosphate or xanthine was not able to increase the formation of formaldehyde in the molybdenum-deficient liver homogenates as it did in the normal homogenates.

3. The utilisation of added formaldehyde was also depressed in the molybdenum-deficient homogenates.

4. The liver xanthine oxidase activity fell to very low values after the feeding of tungstate, but the catalase content of liver was not affected by it.

This work has been aided by a generous grant from the Foundation for the Research of Alcohol Problems.

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Acta physiol. scand. 1959. 45. 116—121.

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# Influence of Some Specific Group Inhibitors on Rat Intrinsic Factor.

By

RALPH GRÄSBECK.

Received 4 September 1958.

**Abstract.**

GRÄSBECK, RALPH. Influence of some specific group inhibitors on rat intrinsic factor. *Acta physiol. scand.* 1959. *45.* 116—121. — The influence of some specific group inhibitors on rat intrinsic factor was studied in order to elucidate the nature of the active structure of intrinsic factor. Iodine and fluorodinitrobenzene inhibited both the vitamin $B_{12}$-binding capacity and the physiological intrinsic factor activity, whereas *p*-chloromercuribenzoate and *di*-isopropylfluorophosphate lacked effect on either activity. The results add further evidence in favor of the concept that intrinsic factor contains an essential structure which binds $B_{12}$, and that a phenolic group may be involved in this structure.

A series of previous reports described the influence of group blocking agents and enzymes on the $B_{12}$-binding capacity (GRÄSBECK 1958 a) and physiological activity (GRÄSBECK 1958 b) of human and hog intrinsic factor material. However, some of the chemical agents being toxic, their action on the physiological activity could not be assayed on patients. The influence of these was therefore studied on gastrectomized rats. For the sake of comparison the influence of iodine was also studied.

## Material and Methods.

Adult Wistar rats were operated on in ether anesthesia. The stomach was removed completely and the esophagus was sewn with silk end-to-side to the duodenum; during this stage of the operation the esophagus was intubated with a thin rubber catheter, around which the stitches were placed. After completion of the anastomosis penicillin-streptomycin powder was strewn over the operation area, and 4 ml of physiological saline was injected into the peritoneal cavity during the closing of the wound. After the operation the rats received no other food than sugar water for 4 days, thereafter milk and gradually more solid food. The rats were used for experiments not earlier than 15 days after the operation. Esophageal intubation was performed a few times before the actual experiments to prevent the formation of strictures. The immediate and late operation mortality was about 60 per cent. When not in use for experiments, the rats received 1 $\mu$g $B_{12}$ parenterally a week. Experiments were not performed earlier than one week after the last injection.

The intrinsic factor activity was assayed in the following manner: Having fasted overnight the rats were given a light ether anesthesia to ensure quantitative administration of the experimental material. A force-feeding tube was passed down the esophagus and the material was injected with a syringe in the following order: 2 ml of the intrinsic factor preparation (or water when no intrinsic factor was given), 1 ml water containing 10 m$\mu$g $\approx$ 10 m$\mu$c of $Co^{60}$-labeled radiovitamin $B_{12}$ ("Merck"), and finally, 1 ml of water to rinse the tube and the syringe. The rats were put into individual metabolism cages and their feces were trapped on wire nets and collected with one or two day intervals for seven days. The feces were put into calibrated "Pyrex" test-tubes, labeled with an ordinary glass-crayon, which leaves a mark visible after incineration, and the tubes were put into an oven kept at 600° C. The incineration of one day's feces took about 24 hours, and thus the material of the whole experimental period was ready to be counted one day after the ending of the collection.

After incineration, the contents of the test-tubes were adjusted to a volume of 7 ml, using dilute hydrochloric acid. The tubes were then counted in a well-type scintillation counter. A tube containing 10 m$\mu$g $B_{12}Co^{60}$ was used as a standard; this gave a count about 13 times the background.

A rat intrinsic factor preparation was produced in the following way: During the killing of normal rats for other purposes the glandular parts of their stomachs were cut out and rinsed quickly with distilled water and stored frozen at — 18° C until used after 2—3 weeks. After thawing, the stomachs were washed with ice-cold 1 per cent sodium bicarbonate solution, under which liquid the mucosa was scraped off with a sharp knife. The scrapings were homogenized and dialyzed against distilled water for three days, after which time the material was lyophilized. The dry material bound about 12 m$\mu$g $B_{12}$/mg, as measured by

adding radiovitamin $B_{12}$ followed by dialysis (GRÄSBECK 1956). A few orientating experiments indicated that when 10 m$\mu$g $B_{12}$ was administered simultaneously, 10 mg stomach powder elicited a better response than 5 mg and that 20 mg gave about the same response as 10 mg. It was therefore decided to administer 10 mg in the actual experiments. This dose also had the advantage that the binding capacity was almost satured when 10 m$\mu$g $B_{12}$ was administered simultaneously.

The effect of the specific group inhibitors was tested in the following manner: All rats were first given only radioactive $B_{12}$. Next, a control intrinsic factor preparation was given, and finally the treated preparation, equal amounts of both preparations being given. The experiments were usually started immediately after the completion of the previous experimental period. For control purposes, the order of administration of the last two doses was reversed in many cases.

The group blocking treatment was done as follows:

*Iodine.* Alcoholic $I_2$ solution was added at 0° C and pH 9 (carbonate buffer) until a final concentration of 0.008 N, the concentration of the stomach extract being 4 mg/ml. Only alcohol was added to the control. Afterwards dialysis at 0° C against pH 9 buffer for 5 hours and later against water to remove free iodine.

*Di-isopropylfluorophosphate.* To an aqueous solution of 10 mg stomach powder per ml was added in one experiment 0.25 mg and in another one 0.5 mg DFP per ml and the preparation was stirred vigorously for 3 hours. Afterwards dialysis against water for 3 days.

*p-chloromercuribenzoate.* The substance was first dissolved in a small volume of dilute sodium hydroxide, the pH of which was subsequently adjusted to 7.4. This was added to a solution of 10 mg stomach powder per ml until a final concentration of 1.7 mg PCMB per ml was achieved. The mixture was allowed to stand for 24 hours at 4° C. The visible PCMB crystals were removed by decantation, and afterwards the preparation was dialyzed against pH 8 bicarbonate buffer for three days.

*Fluorodinitrobenzene.* 30 mg FDNB/ml was added to a solution of 10 mg stomach powder per ml pH 9 phosphate buffer and the solution was stirred vigorously for 3 hours at room temperature. The pH was checked frequently. Afterwards the FDNB was allowed to settle and was removed by decantation. The supernate was dialyzed for 3 days against pH 8.0 phosphate buffer.

The influence of the treatment on the $B_{12}$-binding capacity was assayed by adding radioactive $B_{12}$, followed by dialysis as described by GRÄSBECK (1956).

## Results.

*Effect on $B_{12}$-binding capacity.* Iodine and fluorodinitrobenzene had a clearcut inhibiting effect on the binding capacity, whereas the effect of the two other chemical agents was highly questionable (Fig. 1).

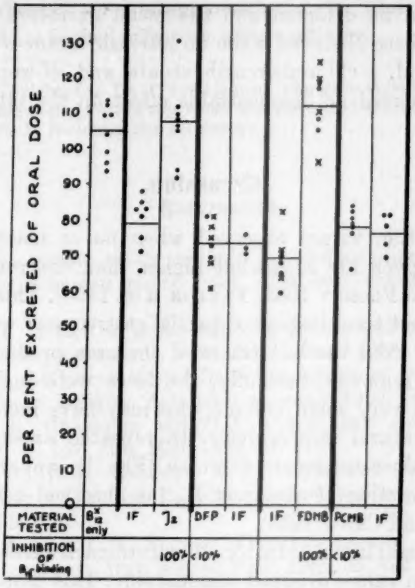


*Fig.* 1. Influence of iodine ($I_2$), *di*-isopropylfluorophosphate (DFP), fluorodinitrobenzene (FDNB), and *p*-chloromercuribenzoate (PCMB) on rat intrinsic factor. The horizontal lines indicate mean values, and the crosses and points refer to different batches of treated stomach extract. IF = Control intrinsic factor preparation.

*Absorption studies.* Absorption of radiovitamin $B_{12}$ alone. 8 normal rats were given 10 $m\mu g$ $B_{12}Co^{60}$ by mouth. They excreted 35—59 % (mean 41.3 %) of the radioactivity with the feces during seven days after injection. The gastrectomized rats excreted between 93 and 113 % (mean 105 %).

Effect of stomach extract. In four series intrinsic factor (*i. e.* the control samples, which had been subjected to the same treatment as those treated with specific group inhibitors, only the specific treatment being omitted) decreased the excretion of radioactive material considerably. There was no overlapping with the values observed when no intrinsic factor was given.

Effect of specific group inhibitors. When the stomach extract was treated with iodine or fluorodinitrobenzene, no intrinsic factor

activity could be detected and the mean excretion values were even above those observed when no intrinsic factor was given. On the other hand, *p*-chloromercuribenzoate and *di*-isopropylfluorophosphate exerted no demonstrable effect on the intrinsic factor activity.

## Discussion.

The excretion values observed when no or inactive intrinsic factor was given are somewhat higher than observed by others (WATSON and FLOREY 1955, TAYLOR *et al.* 1958). This may be due to the fact that total instead of partial gastrectomy was performed and thereby even the last traces of the area producing intrinsic factor were removed. Secondly, the feces were incinerated and counted in a very small volume; this may have increased the influence of natural radioactivity. In repeated experiments these excretion values tended to increase (Fig. 1), apparently due to the fecal excretion of absorbed $B_{12}$, as observed before (OKUDA, GRÄSBECK and CHOW 1958).

When control intrinsic factor (10 mg stomach extract) was given, the excretion values dropped considerably. This drop was of about the same order of magnitude as observed by others (TAYLOR *et al.* 1958), especially considering that more than 100 % was excreted when no or inactive intrinsic factor was administered.

The absence of inhibiting effect of *p*-chloromercuribenzoate on both the $B_{12}$-binding and intrinsic factor activities indicates that a sulfhydryl group is not involved in these processes. The effect of iodination, also observed with human and hog material, and fluorodinitrobenzene support the conclusion outlined previously (GRÄSBECK 1958 a, b) that a tyrosyl group may be of importance. The parallel reaction of both the $B_{12}$-binding capacity and the intrinsic factor activity in the present study adds further evidence in favor of the concept that intrinsic factor contains a structure which binds $B_{12}$ (GRÄSBECK 1956, 1958 a, b).

## Summary.

The influence of some specific group inhibitors on rat intrinsic factor was studied. Iodine and fluorodinitrobenzene inhibited both the $B_{12}$-binding capacity and the physiological intrinsic factor

activity, whereas *p*-chloromercuribenzoate and *di*-isopropylfluorophosphate lacked effect on either activity.

I am greatly indebted to Dr. T. Luukkainen, who gave valuable instruction in the operation technique. Part of this study was performed as student work by the medical students L. Runeberg and K. Simons.

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Acta physiol. scand. 1959. *45*. 122—132.

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# The Estimation of Catechol Amines in Urine.

By

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Received 10 September 1958.

**Abstract.**

EULER, U. S. v. and F. LISHAJKO. The estimation of catechol amines in urine. *Acta physiol. scand.* 1959. *45*. 122—132. — Some modifications in the fluorimetric method of EULER and FLODING are described. The urine sample, usually 25 ml, is adjusted to pH 8.2—8.3 and passed through a column of aluminium oxide. The catechols are eluted with acetic acid, neutralized with ammonia to pH 6.2—6.3, oxidized with potassium ferricyanide and transformed to stable lutines with alkali and ascorbic acid. The fluorescence of the lutines is read with two filter sets and the amounts of adrenaline and noradrenaline computed.

Of the methods described for the quantitative estimation of catechol amines in urine most are based on adsorption on aluminium hydroxide or aluminium oxide at pH 8—8.5, elution with acid and subsequent estimation of the amines in the eluate. This can be achieved either by biological assay, using a suitable pair of test preparations (EULER and HELLNER 1951) or by chromatographic separation and comparison with standards on various test objects (CRAWFORD and LAW 1958). The eluate from aluminium oxide may contain disturbing factors when the bio-

assay is made on the rat's uterus, as recently reported by DILLER (1958).

For the quantitative estimation of the two amines in a mixture also fluorimetric estimation of the corresponding stabilized lutines (EHRLÉN 1948, LUND 1949) has been used. According to LUND (1949), EULER and ORWÉN (1955) and EULER and FLODING (1956) a differentiation of adrenolutine and noradrenolutine is obtained by oxidation at pH 3.5 and pH 6—6.5. The latter method has been modified by SCHAEPDRYVER (1958). Recently PRICE and PRICE (1957) and COHEN and GOLDENBERG (1957 a, b) have determined the lutines in a mixture by using two different filter sets in the fluorimetric estimation after oxidation at pH 6—6.5. By choosing suitable filter combinations the fluorescence ratio between adrenolutine and noradrenolutine can be varied in such a way as to allow a calculation of the amount of the two lutines.

In the method described by EULER and FLODING (1956) the catechol amines are adsorbed from urine by stirring with aluminium oxide at pH 8.5, filtering, washing and elution with sulfuric acid. Of the eluate one sample is oxidized at pH 3.5 and a second sample at 6.0—6.5. From the readings the content of adrenaline and noradrenaline in the eluate can be computed. A draw-back of this method is that it requires oxidation at two different pH which have to be carefully checked. With an acetate buffer at pH 6.5 the risk of obtaining a lower pH than 6.0, which results in incomplete oxidation of the noradrenaline, is reduced but still requires attention when larger volumes of the eluate are used. The recovery of added adrenaline and noradrenaline is generally about 70 per cent, but may on some occasions, which seem difficult to control, be markedly lower.

In order to improve the recovery of the catechol amines and increase the precision of the method some modifications have been introduced which will be described in the present paper.

## Experimental.

*Adsorption.*

In most instances an amount of 25 ml urine was found suitable. Smaller volumes may be conveniently diluted to 25 ml. When the diuresis is considerably increased above normal, larger amounts may be used. After boiling and filtering the urine, 0.5 g of the di-sodium salt of ethylene diamine tetraacetate (Titriplex) is

added. The urine is then adjusted to pH 8.2—8.3 with 1.0 N NaOH which is added dropwise under stirring. At this pH the catechol amines in urine are stable for at least 1 hour.

The aluminium oxide used for adsorption is heated with 2 N hydrochloric acid under stirring during 20 min at 100° C, thoroughly washed with distilled water to pH 4.0 and dried. Of the alumina 1 g is suspended in distilled water and poured into a column of 10 mm bore with a sintered glass filter above the stopcock at the lower end. The stem of the column is 15 cm, having an upper wider part holding about 50 ml. In order to prevent clogging up, the glass filter is covered with a snugly fitting disc of filterpaper. The aluminium oxide is allowed to settle and air bubbles removed by gentle stirring with a glass rod. The water is allowed to drain to the upper surface of the alumina column and another filter paper disc placed on top of the alumina column which is about 2 cm high.

The urine at pH 8.2—8.3 is carefully added to the column and allowed to pass through the alumina at a rate of 1—2 ml per min. After the urine has passed the column it is washed with 10—15 ml distilled water at the same rate. The adsorption during one passage as described was found to be complete for practical purposes, in that less than 5 per cent of originally present or added catechol amines were found in the urine after passage.

*Elution.*

The catechol amines are eluted with 0.25 N acetic acid to the column. After addition of 5 ml acid the alumina suspension is briefly stirred with a glass rod. The eluting acid is then allowed to pass at a rate of 1—2 ml per min. Complete elution is achieved by passing another 5 ml acetic acid through the column. This was tested by using standard solutions of adrenaline and noradrenaline to which ascorbic acid 0.1 per cent had been added. The total eluate has a pH-value of about 3.

The catechol amines in the eluate are stable for several hours at room temperature and for longer time when kept frozen.

*Formation of lutines.*

Before oxidation the eluate is neutralized to pH 6.2—6.3 with 1 N ammonia which is added dropwise under stirring. After

neutralization the volume of the eluate is measured and the oxidation carried out within 15—30 min in an aliquot.

The catechol amines are transformed into fluorescing lutines by oxidation with potassium ferricyanide followed by tautomerization by strong alkali in the presence of ascorbic acid as stabilizing agent (EHRLÉN 1948, LUND 1949). Zinc sulphate did not increase the yield.

Depending on the catechol amine content of the eluate, and the sensitivity of the fluorimeter a suitable volume is used for the oxidation to the corresponding chromes by addition of 0.1 ml 0.25 per cent potassium ferricyanide which is allowed to act for 2 minutes. After this time 1 ml of a freshly prepared mixture of 18 ml 20 per cent sodium hydroxide and 2 ml 2 per cent ascorbic acid is added to the eluate, causing immediate transformation of the chromes to the corresponding lutines in a stabilized form. After mixing, water is added to 10 ml within 1/2 minute, and thoroughly mixed. When larger volumes of eluate than 1 ml are used, an equal volume of the alkali-ascorbic acid solution is added. The fluorescence is measured within 10 min.

*Blank.*

The blank is prepared by adding all reagents except the potassium ferricyanide to the same volume of eluate as used in the sample. By applying the filter combinations suggested by COHEN and GOLDENBERG (1957 b) the blank figures could be kept low. Tendencies to increased blank values indicate faulty technique or impure or aged reagents.

*Fluorimetric estimation.*

The fluorimeter used was Coleman Model 12 C which was found stable and suitable for routine estimations. The filter combinations used were the same as employed by COHEN and GOLDENBERG (1957 b). Filter set A consisted of a primary interference filter 395 m$\mu$ for excitation combined with an Ilford Bright 623 secondary filter (peak transmission 490 m$\mu$). Using this filter combination adrenolutine and noradrenolutine gave nearly equal fluorescence figures.

Filter set B was composed of a 436 m$\mu$ interference filter for excitation and a Corning 3486 secondary filter giving peak trans-

$$\text{Noradrenaline} = y = \frac{(mA_b/A_a) - n}{N_aA_b/A_a - N_b}$$

$$\text{Adrenaline} = x = \frac{n - yN_b}{A_b}$$

mission at 540 m$\mu$. With this filter set adrenaline gives about 3 times as high fluorescence as noradrenaline.

The fluorescence of the sample and the blank were determined with both filter sets, and the amounts of adrenaline and noradrenaline calculated with the aid of the following formula (COHEN and GOLDENBERG 1957 b).

In the equations, $m$ and $n$ represent the actually observed fluorescence values (sample minus blank), using filter sets $a$ and $b$ respectively. $A_a$, $A_b$, $N_a$ and $N_b$ represent the values for the fluorescence per $\mu$g adrenaline and noradrenaline respectively at the same sensitivity as for the samples using filter sets $a$ and $b$. For convenience a lucite rod inserted in a test tube is used as standard after determination of its fluorescence value in terms of adrenaline and noradrenaline.

A rectilinear relationship was found to obtain between the fluorimetric readings and the amounts of standard solutions of catechol amines added, in conformity with the observations of COHEN and GOLDENBERG (1957 b). Using the standard constants for adrenaline and noradrenaline the amounts added and those obtained by computing the figures according to the formula showed good agreement (Table I).

The sensitivity of the apparatus can be considerably increased by the use of the Coleman 12 C Galv-O-Meter outfit which was found useful when the lutine concentration in the eluate was low.

*Recovery of added adrenaline and noradrenaline.*

In a series of tests adrenaline and noradrenaline were added to the urine and the recovery estimated after carrying the added amines through the entire procedure (Table II).

When amounts of 0.25—2 $\mu$g of either amine were added to urine in varying proportions the recovery was from 68 to 100 per cent in all cases except one (51 per cent) as seen in the table. A consistent recovery of this order may be regarded as satisfactory for most purposes.

The method showed a reasonable good degree of precision.

**Table I.**

*Added and estimated catechol amines in mixtures in μg.*

| Added | | Estimated | |
|---|---|---|---|
| Adr. | Noradr. | Adr. | Noradr. |
| 0.50 | 0.50 | 0.52 | 0.48 |
| 0.20 | 0.20 | 0.190 | 0.190 |
| 0.40 | 0.20 | 0.399 | 0.205 |
| 0.20 | 0.40 | 0.21 | 0.41 |
| 0.20 | 0.30 | 0.185 | 0.295 |

**Table II.**

*Recovery of adrenaline and noradrenaline added to urine.*

| Added to urine (μg) | | Recovered in per cent | |
|---|---|---|---|
| A | NA | A | NA |
| 0.25 | 0.25 | 68 | 96 |
| 0.50 | 0.50 | 72 | 90 |
| 1.0 | 1.0 | 79 | 96 |
| 0.25 | 1.0 | 100 | 85 |
| 1.0 | 0.25 | 68 | 100 |
| 0.25 | 1.0 | 88 | (51) |
| 0.50 | 0.50 | 82 | 72 |
| 1.0 | 0.25 | 78 | 91 |
| 0.25 | 0.25 | 96 | 72 |
| 1.0 | 1.0 | 88 | 68 |
| 2.0 | 2.0 | 81 | 68 |

This will undoubtedly depend to some extent on the individual technique since the procedure involves a large number of steps. In 5 samples of the same urine, carried through the complete procedure as described, the adrenaline figures per 25 ml were 0.41—0.44 μg and the noradrenaline figures 1.48—1.56.

*Catechol amine excretion in urine in healthy subjects during day and night hours.*

The excretion of catechol amines in urine was determined in day and night urine in 5 healthy subjects (Table III) and in two cases of phaeochromocytoma.

The figures in Table III compare well with those previously

**Table III.**

*Adrenaline and noradrenaline excretion in urine in 5 healthy subjects during day and night hours.*

| Subject | Spec. gravity | Average urine excretion ml/min | Excretion ng/min | | Remarks |
|---|---|---|---|---|---|
| | | | A | NA | |
| 1..... | 1.012 | 1.9 | 6.1 | 28 | Day urine |
| 2..... | 1.020 | 0.72 | 17 | 25 | » » |
| 3..... | 1.027 | 0.48 | 11 | 27 | » » |
| 4..... | 1.023 | 0.41 | 11 | 30 | » » |
| 5..... | 1.006 | 2.5 | 7.3 | 17 | » » |
| Average ... | 1.018 | 1.20 | 10.5 | 25 | |
| Range ..... | (1.006—1.027) | (0.41—2.5) | (6.1—17) | (17—30) | |
| 1..... | 1.017 | 0.21 | 0.60 | 3.5 | Night urine |
| 2..... | 1.021 | 0.56 | 4.3 | 13 | » » |
| 3..... | 1.026 | 0.32 | 0.74 | 8.7 | » » |
| 4..... | 1.022 | 0.41 | 0.61 | 11 | » » |
| 5..... | 1.015 | 0.78 | 3.7 | 11 | » » |
| Average ... | 1.020 | 0.46 | 2.0 | 9.4 | |
| Range ..... | (1.015—1.026) | (0.21—0.78) | (0.60—4.3) | (3.5—13) | |

reported by EULER and ORWÉN (1955) and by KÄRKI (1956) and PITKÄNEN (1956). Subject 2 who showed the highest adrenaline figures both during night and during day hours was a fairly heavy smoker in difference to the other subjects. It has been reported (WATTS and BRAGG 1956) that smoking increases the adrenaline excretion in urine.

Two cases of phaeochromocytoma, verified by operation, showed noradrenaline excretion figures varying between 282 and 560 ng/min in one case and 320 ng/min in the second.

## Discussion.

A large number of modifications have been tried before the procedure outlined above was adopted. Some of these will be briefly mentioned.

*Aluminium oxide.* Aluminium oxide British Drug Houses gave satisfactory yield even without acid treatment. Previous passage of sodium acetate buffer through the column did not seem to offer advantages. The adsorption on a column gave somewhat better recovery of added catechol amines than stirring with aluminium oxide, whether this was added at pH 3 or pH 8.5.

Better yields were not obtained with 1.5—3.5 g aluminium oxide than with 1 g.

*Urine.* Higher yields were obtained when 25 ml urine was used for absorption than if 50 or more ml were used. Dilution of the urine did not consistently increase the yield, nor was this the case after washing the alumina after adsorption with 50—200 ml water instead of 25 ml.

No difference in the yield and recovery was observed when urine was alkalinized before adsorption with 0.1 or 0.5 N NaOH or weaker bases, than with 1 N NaOH, only the alkalinization was made dropwise and under stirring.

Titration of urine to pH 8.0 gave slightly less yield and recovery than to pH 8.5. At pH 9.0 the recovery was markedly diminished.

Addition of ethylene diamine tetraacetic acid was found to be essential for a good yield. Addition of 0.5 g to 25 ml urine gave equally good results as addition of 1.5 and 2 g.

Addition of 10—1,000 mg glycine or 3—30 mg ascorbic acid per 100 ml urine did not improve the yield. Larger amounts of ascorbic acid decreased the recovery of added amines.

*Elution.* Brief stirring of the alumina with 5 ml 0.25 N acetic acid and addition of a second 5 ml (without stirring) after the first 5 ml had passed, was found to increase the yield and recovery. After mixing of the acid with the alumina the elution process is rapid, the yields being equally good after 1/2 min as after 5 min. No difference in yield was noted when 0.25 N or 1 N acetic acid was used. Sulfuric acid seems to elute more disturbing substances than acetic acid but has the advantage that it can be directly tested (after neutralization) on many biological test preparations in difference to the acetic acid eluates.

*Fluorimetric estimation.* Neutralization of the eluate with 0.5—1 N NaOH to pH 6.0—6.5 caused considerable losses and increased the blank values. These could be minimized by using 0.5—1 N ammonia or 1 M sodium triphosphate or sodium hydrogen carbonate for adjusting pH of the eluate to 6.0—6.5.

The amount of potassium ferricyanide used for oxidation did not seem to be critical. Thus 0.1—0.4 ml of the 0.25 % solution could be used without any differences in yield being noted. If oxidation time was increased from 2 min to 3 min this resulted in reduced yield, however. The amounts of sodium hydroxide-ascorbic acid have to be increased according to the volume of eluate used for the estimation, presumably on account of its

buffering capacity. This seems to be particularly important for the recovery of noradrenaline and less so for adrenaline. As a general rule equal volumes of sodium hydroxide-ascorbic acid mixture and eluate gave satisfactory results. Immediate thorough mixing of the eluate with the sodium hydroxide-ascorbic acid mixture was also found to be essential.

*Recovery.* In the attempts to improve the method for the quantitative estimation of adrenaline and noradrenaline in urine, attention has been paid not only to the recovery of added amounts of catechol amines but also to the yield of catechol amines originally present in urine. Using the technique described the highest figures have been obtained when compared with the yield using various modifications of the method. It is not certain that the recovery of added catechol amines always runs parallel with the yield of amines already present in urine. The use of "internal standards" may give useful hints but these should not be taken as giving a quantitative indication of the yields of the amounts originally present.

*Other catechol amines.* Urine contains, in addition to noradrenaline and adrenaline, also dopamine, as first shown by HOLTZ, CREDNER and KRONEBERG (1947). Using the method of estimation described in the present paper dopamine gives only 1—3 per cent of the fluorescence obtained for the same amount of noradrenaline. Since the disturbing action of dopamine in the concentrations usually occurring in urine is considered as negligible no attempts have been made to separate dopamine from the other amines in urine. If iodine is used as oxydant, dopamine may cause gross interference, however (SHORE and OLIN 1958).

Isopropylnoradrenaline (isoproterenol) would theoretically have a much greater influence since it gives a fluorescence of similar magnitude as noradrenaline and adrenaline. However, the amounts present in urine are usually extremely small if the amine is occurring in urine at all. It has been reported, however, that on rare occasions isoproterenol occurs in urine (SUBRAHMANYAM 1958).

## Summary.

1. An improved fluorimetric method for the quantitative estimation of adrenaline and noradrenaline in urine is described.

2. The method is based on adsorption of the catechol amines

on an aluminium oxide column at pH 8.2—8.3 and elution with 0.25 N acetic acid.

3. After neutralization of the eluate with ammonia, the lutines are obtained by oxidation at pH 6.2—6.3 with ferricyanide and transformation by strong alkali and ascorbic acid according to EHRLÉN and LUND.

4. Adrenaline and noradrenaline are estimated by the fluorescence of their lutines, using two filter sets according to COHEN and GOLDENBERG.

5. The recovery of added catechol amines is 70—100 per cent.

This study has been supported by a grant from the Rockefeller Foundation to U. S. v. E. For valuable discussion the authors are indebted to Dr. R. Eliasson.

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# Some Biological Effects of Two Crystalline Prostaglandin Factors.

By

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Received 16 September 1958.

**Abstract.**

BERGSTRÖM, S., R. ELIASSON, U. S. v. EULER and J. SJÖVALL. Some biological effects of two crystalline prostaglandin factors. *Acta physiol. scand.* 1959. *45*. 133—144. — The effects of two crystalline fractions of prostaglandin (PGE and PGF) were tested on a variety of isolated organs and on the blood pressure of the rabbit. Characteristic differences in the activity ratio for PGE and PGF were noted for different organs. The biological actions of partially purified preparations of prostaglandin could be largely but not wholly explained by assuming that they contained a mixture of PGF and PGE.

Extracts of human seminal plasma and of the vesicular gland of sheep, prepared with lipid solvents, produce a fall in blood pressure in various experimental animals and a stimulating action on a variety of smooth muscle organs (GOLDBLATT 1933, 1935, EULER 1934, 1935, 1936, 1939). The active preparation, called prostaglandin, was shown to have acidic properties (EULER 1935). Further studies have indicated that the activity is of a similar kind

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in extracts of human seminal plasma and in the vesicular gland of sheep (ELIASSON 1957).

Previous studies suggested that the active principle was an unsaturated hydroxy acid (EULER 1939, BERGSTRÖM 1949). Recently two crystalline compounds have been isolated from the vesicular gland of sheep, both being nitrogen free, unsaturated acids (BERGSTRÖM and SJÖVALL 1957, 1959). The present report deals with the effects of these two compounds (PGE and PGF) on the blood pressure of the rabbit and on various smooth muscle preparations from different animals.

## Methods.

Prostaglandin E and F (PGE and PGF) had been prepared according to the method described by BERGSTRÖM and SJÖVALL (1959). The pure substances were dissolved in sterile saline and kept frozen until tested. Particular care had to be observed as regards Prostaglandin E which tended to become inactivated in solution at room temperature.

The tests were made on the blood pressure of the rabbit, anaesthetized with urethane, and on the following isolated organs: jejunum (rabbit, chicken, rat), ileum (guinea-pig), uterus (rabbit, guinea-pig, rat) rectal caecum (chicken), iris (cow). The isolated organs were mounted in the conventional way in a 3—15 ml bath with Tyrode solution at 38° C., aerated with 5 per cent carbon dioxide in oxygen, or with pure oxygen in the case of the guinea-pig ileum.

Atropine sulphate and phenergan 1—2 $\mu$g per ml were added in some experiments, but since these additions did not alter the response they were omitted in the majority of the tests.

In a number of experiments the actions of the isolated preparations E and F were compared with a barium salt preparation previously used (EULER 1939) and with a purified extract of vesicular gland of sheep (ELIASSON 1957). This latter preparation is designed RE in the following.

## Results.

*Rabbit blood pressure.*

Preparation E caused the typical fall in blood pressure when injected i. v. in the rabbit. Even doses as small as 0.5 $\mu$g produced a definite effect. Preparation F, on the other hand, had no action, even in doses of 10 $\mu$g (Fig. 1). The effect of prostaglandin E was not prevented by doses of atropine or antihistaminics sufficient to abolish the action of acetylcholine or histamine. The effects were of the same fairly long-lasting character as noted for seminal plasma or partly purified preparations.

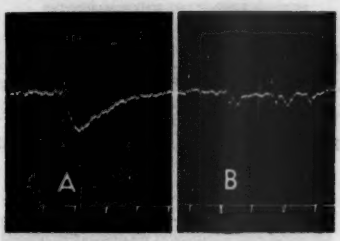


Fig. 1. Blood pressure. Rabbit. Urethane.
A: 1.5 μg PGE,
B: 10 μg PGF.

*Rabbit jejunum.*

This preparation has been extensively used in testing prostaglandin preparations in presence of atropine.

In two of the six experiments PGE and PGF had about similar activity per unit of weight, while in the four others PGF had a stronger action which on one occasion was about 3 times that of PGE (Fig. 2). A certain difference in the type of action was also noted in that PGE usually caused a brisk increase in the contraction height, whereas PGF as a rule caused a gradual gain in amplitude.

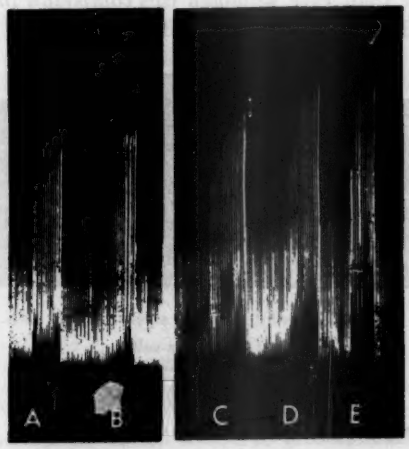


Fig. 2. Isolated jejunum, rabbit. 15 ml bath.
A: 0.3 μg PGE,
B: 0.1 μg PGF,
C: 2 μg Ba-salt,
D: 0.02 unit RE,
E: 0.5 μg PGE.

*Rat jejunum.*

On the rat jejunum PGE always had a markedly stronger action than PGF. It was also noted that PGE in some cases caused a well marked inhibition in tone and amplitude before the stimulating effect. The primary inhibition was not noted with PGF (Fig. 3.)

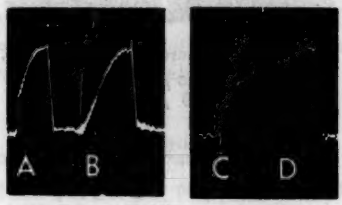


Fig. 3. Isolated jejunum, rat. 3 ml bath.
A: 2 μg PGF,
B: 0.07 unit RE,
C: 0.05 unit RE,
D: 0.15 μg PGE.

*Chicken jejunum.*

As in the rat jejunum PGE had a considerably stronger stimulating effect than PGF although this compound in higher doses (5 μg) caused a weak but definite effect (Fig. 4).

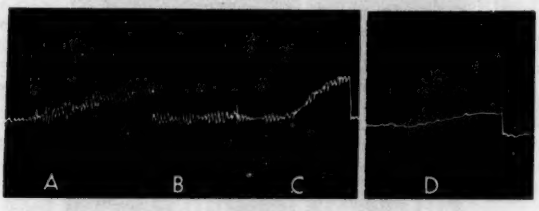


Fig. 4. Isolated duodenum, chicken. 15 ml bath.
A: 6 μg Ba-salt,
B: 1 μg PGF,
C: 0.5 μg PGE,
D: 5 μg PGF.

*Guinea-pig ileum.*

PGE exerted a strong stimulating effect on the tone of the guinea-pig ileum which was 20—70 times that of PGF (Fig. 5).


Fig. 5. Isolated ileum, guinea-pig.
15 ml bath.
A: 5 μg PGF,
B: 0.1 μg PGE,
C: 0.07 μg PGE.

*Chicken rectal caecum.*

This preparation, originally introduced by BARSOUM and GADDUM (1935) showed a good response to both PGE and PGF, the latter substance being the more active in contrast to the effects on the chicken jejunum. The effect was long-lasting (Fig. 6).

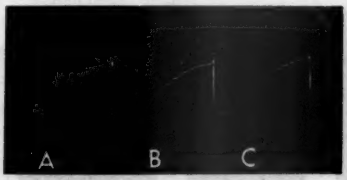


Fig. 6. Isolated rectal caecum, chicken.
15 ml bath.
A: 1 μg PGE,
B: 0.5 μg PGF,
C: 0.03 unit RE.

*Rabbit uterus.*

The responses of the rabbit uterus to prostaglandin were fairly irregular. In 2 exp., on ovarectomized stilbol-treated animals, PGE was without action or had a weak action except in one case on the first addition, while PGF consistently stimulated the uterus

in doses less than 1 $\mu$g (Fig. 7). On a pregnant animal PGE had a marked effect in a dose of 1.5 $\mu$g, whereas PGF had no action in a dose of 5 $\mu$g. Untreated, non-pregnant animals either responded to PGE and PGF as the ovarectomized rabbits or showed inhibition of the uterine movements.

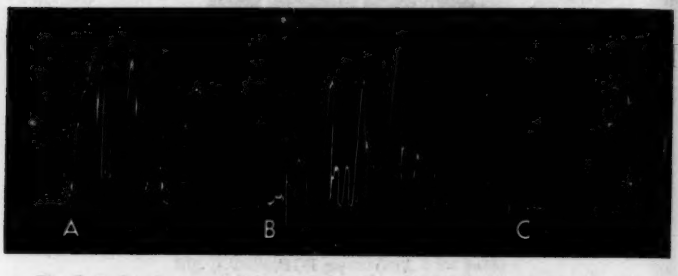


Fig. 7. Isolated uterus rabbit, ovarectomized, stilbol-treated. 15 ml bath.
A: 0.75 $\mu$g PGF,
B: 0.1 unit RE,
C: 2 $\mu$g PGE.

*Guinea-pig uterus.*

On the non-pregnant, non-virgin guinea-pig uterus PGE was about 3 times more active than PGF (Fig. 8).

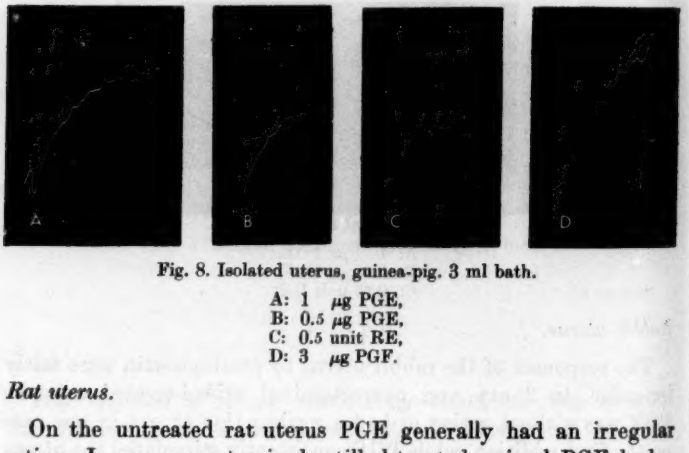


Fig. 8. Isolated uterus, guinea-pig. 3 ml bath.
A: 1 $\mu$g PGE,
B: 0.5 $\mu$g PGE,
C: 0.5 unit RE,
D: 3 $\mu$g PGF.

*Rat uterus.*

On the untreated rat uterus PGE generally had an irregular action. In one ovarectomized, stilbol-treated animal PGF had a

definite action in a dose of 0.1 $\mu$g, while no effect was observed with a 20 times larger dose of PGE.

*Cow iris.*

The isolated iris ring of the cow was quite sensitive to PGE and at the same time almost wholly insensitive to PGF even in large doses. As shown in Fig. 9 0.2 $\mu$g PGE had a well marked slow stimulating action while 5 $\mu$g PGF was without action.

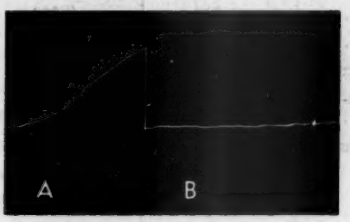


Fig. 9. Isolated iris, cow. 15 ml bath.
A: 0.2 $\mu$g PGE,
B: 5 $\mu$g PGF.

*Relative biological activity of PGE and PGF.*

Table I gives an account of the relative activity of PGE and PGF on the various test preparations used in the present study. As seen in the table very large differences in activity occur, which, when expressed as activity ratio (PGE/PGF), vary from less than 0.5 to 70. As commented upon in the discussion part this circumstance offers a useful basis for differential estimation of PGE and PGF in mixtures.

*Relative activity of PGE, PGF and a standard barium-salt preparation.*

Some of the previous purification experiments were made on a Ba-salt prepared from sheep vesicular gland (EULER 1939, BERGSTRÖM 1949). In some instances the activity of the Ba-salt was compared with that of the pure substances PGE and PGF. The Ba-salt was converted into the sodium salt before testing (Table II).

**Table I.**

*Activity ratio PGE/PGF on different test preparations and approximate threshold doses in μg per ml bath volume.*

| Test preparation | Activity ratio PGE/PGF | Threshold dose in μg | |
|---|---|---|---|
| | | PGE | PGF |
| Rabbit, B. P. | > 20 | 0.05 | > 10 |
| Rabbit jejenum | 0.33—1 | 0.003—0.01 | 0.003—0.01 |
| Rat, jejunum | 5—20 | 0.01—0.03 | 0.08—0.3 |
| Chicken, jejunum | 40 | 0.008 | 0.3 |
| Guinea-pig, ileum | 20—70 | 0.01—0.02 | 0.3—1 |
| Chicken, rectal caecum | 0.5—3 | 0.007—0.03 | 0.01 |
| Rabbit, uterus (non-pregnant) | < 0.5 | > 0.03 | 0.15 |
| Rabbit, uterus (ovarect., stilbol-treated) | 0.08 | 0.4 | 0.03 |
| Rabbit, uterus (pregnant) | ⩾ 3 | 0.5 | ⩾ 1.5 |
| Rat, uterus (non-pregnant) | 0.5 | 0.1 | 0.05 |
| Guinea-pig, uterus | 3 | 0.07 | 0.2 |
| Cow, iris | > 30 | 0.01 | > 0.3 |

**Table II.**

*Comparison of activity of Ba-salt and PGE and PGF on various test preparations.*

| Preparation | Activity of 1 μg Ba-salt (as sodium salt) in terms of | |
|---|---|---|
| | PGE μg | PGF μg |
| Rabbit blood pressure | 0.0083 | > 0.16 |
| Rabbit, jejunum | 0.045 | 0.045 |
| Rat, » | 0.025 | 0.5 |
| Chicken, » | 0.025 | 1 |
| Guinea-pig, ileum | 0.025 | 0.075 |
| Chicken rectal caecum | 0.025 | 1.5 |

The activity relationships between the activity of the Ba-salt, PGE and PGF as shown in Table II suggest that if the activity of the Ba-salt were due to a mixture of PGE and PGF these

**Table III.**

*Activity of a partially purified prostaglandin preparation RE from sheep vesicular gland in relation to the activity of PGE and PGF on different test preparations.*

| Test preparation | Activity of 1 unit prostaglandin RE in terms of | |
|---|---|---|
| | PGE $\mu$g | PGF $\mu$g |
| Rabbit, B. P. | 10—15 | > 200 |
| Rabbit jejunum | 6.6—20 | 6—13 |
| Rat jejunum | 2.5—10 | 30—200 |
| Guinea-pig ileum | 1.3—1.7 | 26—100 |
| Chicken rectal caecum | 33 | 17 |
| Rabbit uterus (non-pregnant) | 2 | 1 |
| » » (ovarect. stilbol-treated) | 40,100 | 8, 10 |
| » » (pregnant) | 1 | 3 |
| Rat uterus (non-pregnant) | 10 | 5 |
| Guinea-pig uterus | 1 | 3 |

should occur in about equal parts (1 $\mu$g Ba-salt appr. = 0.02 $\mu$g PGE + 0.02 $\mu$g PGF).

*Activity of a standard preparation RE from sheep vesicular gland in terms of PGE and PGF.*

As mentioned above a comparison between the activity of prostaglandin preparations from human seminal fluid and sheep vesicular glands showed similar biological properties and approximately the same inactivation at different pH. They also showed a similar paper chromatographic behaviour (Eliasson 1957). It therefore seemed of interest to compare the effects of a standard preparation "RE" from sheep's vesicular gland containing the natural combination of the active factors with the pure fractions PGE and PGF. Table III shows the activity relationships.

## Discussion.

A comparison of the effects of PGE and PGF shows in several instances the same kind of action although there are rather marked quantitative differences. This is illustrated numerically by the

large variations in activity ratios which vary from less than 0.1 to above 50. No action could be elicited on the rabbit's blood pressure with the highest doses of PGF used (10 $\mu$g), and on the cow's iris doses of 5 $\mu$g had no effect. A striking difference in the relative activity of PGE and PGF was noted between rat's jejunum and uterus of which the latter preparation was more sensitive to PGF than to PGE, while the opposite is true for the jejunum. A marked difference was also observed in the relative sensitivity to PGE and PGF on the chicken jejunum and rectal caecum.

The difference in response to PGE and PGF offers a possibility to determine the amount of the two factors differentially in a mixture, using two test preparations with sufficient difference in activity ratio, according to the same principle as utilized for the differentiation of adrenaline and noradrenaline (EULER 1948). This method may be applied under the condition that only or chiefly PGE and PGF are present in the mixture.

Judging from the results of evaluating a purified extract of vesicular gland of the sheep (RE) in terms of PGE and PGF, the natural product, obtained by extraction with ether may contain PGF as well as PGE. Owing to the greater activity of PGE on most test preparations the effect of PGF would become relatively more evident only on the chicken rectal caecum and the uterus of non-pregnant or ovarectomized and stilboltreated rats and rabbits.

The present study has confirmed the results of previous investigations that prostaglandin is a potent stimulant of a variety of smooth muscle organs (EULER 1936, ELIASSON 1957) and lowers the blood pressure particularly of the rabbit. In addition it has been shown that many of these actions can be reproduced by one or both of the factors isolated by BERGSTRÖM and SJÖVALL (1957) in crystalline form, PGE and PGF. Whether or not yet another factor, or factors, occur in crude extracts of the active material from the vesicular gland of the sheep cannot at present be stated with certainty, although some results are suggestive of this. Thus both PGE and PGF inhibited the isolated rabbit uterus in some instances while preparation RE still had a stimulating effect. Also the effects of preparation RE on the rabbit's blood pressure and on the guinea-pig's uterus cannot readily be expressed in terms of PGE and PGF.

## Summary.

1. Two isolated fractions of prostaglandin from the vesicular gland of the sheep, PGE and PGF, have been tested on various test preparations.

2. PGE lowers the blood pressure of the rabbit in doses from 0.5 $\mu$g. No action was observed with PGF in doses up to 10 $\mu$g.

3. Stimulating actions were observed with PGE and PGF on the isolated intestine of rabbit, rat, guinea-pig and chicken, the isolated uterus of the guinea-pig and on the isolated iris muscle of the cow.

4. The activity ratio PGE/PGF showed wide variations on these test preparations, attaining values of less than 0.1 to above 50, allowing differential estimation of PGE and PGF in mixtures on a suitable pair of test objects.

5. The actions of a partially purified preparation as Ba-salt from the vesicular gland of the sheep and a standard extract were compared with those of PGE and PGF.

This work has been supported by grants from "Stiftelsen Therese och Johan Andersons Minne" and "Stiftelsen Gustaf och Tyra Svenssons Minne" to S. B.

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# Observations on the Effects of Infusion of Prostaglandin E in Man.

By

S. BERGSTRÖM, H. DUNÉR, U. S. v. EULER, B. PERNOW and J. SJÖVALL.

Received 16 September 1958.

**Abstract.**

BERGSTRÖM, S., H. DUNÉR, U. S. v. EULER, B. PERNOW and J. SJÖVALL, Observations on the effects of infusion of prostaglandin E in man. *Acta physiol. scand.* 1959. *45*. 145—151. — Chemically pure prostaglandin E was infused in doses of 0.2—0.7 μg/kg/min in two healthy male subjects over periods of 4—10 min. Tachycardia, reddening of the face, headache and an oppressive feeling in the chest were noted. Systemic arterial blood pressure and cardiac output fell moderately.

The pharmacodynamic effects of extracts of seminal fluid and male accessory glands, independently observed by GOLDBLATT (1933, 1935) and by EULER (1934) have been shown to depend on a lipid-soluble factor of acid character (EULER 1935, 1936, 1939). Some of its circulatory actions have been studied in animal experiments in which it was shown that the active principle, called prostaglandin, caused a prolonged fall in blood pressure on intravenous injection in the rabbit and in the cat (EULER 1939). No action was observed on the heart-lung preparation of the cat.

Vasodilator effects were demonstrated in the perfused hind legs of the cat while vasoconstrictor effects were noted in the isolated perfused kidney and lungs of the cat and in human placenta.

Two active compounds have recently been isolated in crystalline form from the vesicular glands of sheep (BERGSTRÖM and SJÖVALL 1957, 1959), of which one (prostaglandin E, "PGE") lowered the blood pressure in addition to its smooth muscle stimulating actions, while the other (prostaglandin F) stimulated smooth muscle organs but had no effect on the blood pressure of the rabbit even in high doses (BERGSTRÖM *et al.* 1959).

In the present report some actions in man of intravenously infused PGE will be described.

## Material and methods.

The study was made on two male healthy subjects aged 21 and 23 years, resting in supine position. A heart catheter was inserted through a cubital vein and the pressures recorded from the right atrium and ventricle, the pulmonary artery and the PCV position. A polyethylene catheter was inserted percutaneously in one brachial artery according to the technique described by BERNÉUS *et al.* (1954) for pressure recordings and collection of blood samples.

The cardiac output was determined by Fick's direct method. Oxygen content and capacity of blood samples was determined spectrophotometrically according to the method of DRABKIN and SCHMIDT (1945) as described by HOLMGREN and PERNOW (1958). The error of the method was about 0.1 ml oxygen per 100 ml blood.

Chemically pure PGE, 2 mg per ml in ethanol, was diluted with sterile saline to 0.01—0.02 mg/ml. The solution was administered intravenously by constant infusion into a cubital vein at a rate of 13—47 $\mu$g per min during 4—10 min.

## Procedure.

The right ventricular, pulmonary artery, PCV, and brachial artery pressures were recorded and the cardiac output determined initially. PGE in concentrations of 10 and 20 $\mu$g per ml respectively was then infused. During these periods the cardiac output was again determined and the pressures recorded repeatedly. The oxygen consumption was measured over a 5 min period, starting as soon as the subjects reported subjective feelings, which occurred about two minutes after the start of the infusions. The in-

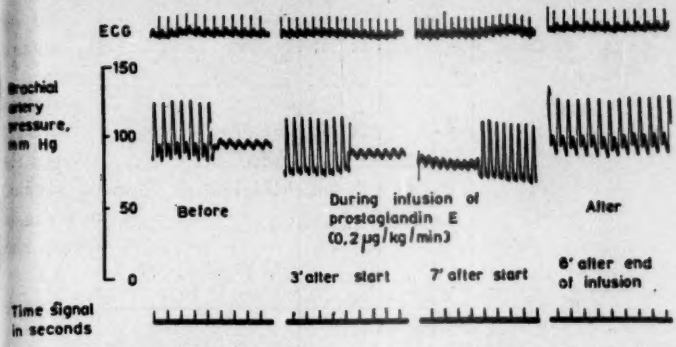


Fig. 1. Upper curve, ECG; lower curve, brachial artery pressure; at bottom, time signal in seconds. A before, B—D 3, 7 and 8 min after start of i. v. infusion of prostaglandin E 0.2 µg/kg/min in a healthy young male subject.

fusion was thereafter repeated at an increased rate up to 47 µg per minute, with observations on the general symptoms and continuous blood pressure recordings.

## Results.

*General symptoms.*

Two to three minutes after the start of the infusion at a rate of 13—15 µg of PGE per min the subjects got a feeling of warmth and oppression in the head and chest and flushing in the face. These symptoms remained during the whole infusion. When the infusion rate was increased to about 20 µg per min the symptoms became more pronounced with a bright red flush of the face, accompanied by an intense feeling of warmth. A feeling of pressure in the head and constriction of the larynx and thorax and palpitations of the heart were also noted. These symptoms were still present some 5—10 min after the end of infusion. Higher doses of PGE (47 µg per min) were tolerated only about 5 min. Already one minute after the beginning of the infusion the subject complained of pulsations in the head with severe headache, oppression in the chest and severe general discomfort.

*Pulse rate.*

During infusion of PGE the pulse rate increased varying from 10 to 24 strokes per min. The frequency increased continuously during the infusion (Fig. 1, Table I). About 15 min after

**Table I.**

*Pulse rate, blood pressure (mm Hg), cardiac output (l/min) and stroke volume (ml) before, during and after infusion of prostaglandin E (PGE) in a single experiment.*

| Resting value before PGE | | Pulse rate | Right ventricle | | Pulmonary artery | | | PCV | Brachial artery | | | Cardiac output | Stroke volume |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | systolic | end-diastolic | systolic | diastolic | mean | mean | systolic | diastolic | mean | | |
| | | 80 | 18 | 1 | 19 | 4 | 7 | 8 | 137 | 86 | 105 | 10.1 | 126 |
| | | | | | | | | | | | | 10.3 | 128 |
| Infusion of PGE 15 μg per min | 1½ min | 84 | 18 | 1 | | | | 7 | 127 | 83 | 97 | | |
| | 3 min | 94 | 17 | 1 | | | | | 121 | 77 | 94 | 8.4 | 90 |
| | 5 min | 94 | 19 | 1 | 18 | 4 | 6 | 7 | 119 | 72 | 88 | | |
| | 7 min | 104 | 21 | 0 | | | | | 112 | 55 | 85 | | |
| After infusion of PGE | 3 min | 97 | | | | | | | 121 | 75 | 102 | | |
| | 8 min | 92 | 20 | 0 | 18 | 4 | 7 | | 130 | 88 | 105 | | |

the end of the infusion the starting-point level of the pulse rate was again reached.

*Blood pressure.*

No marked changes were observed in the pressure in the right ventricle, pulmonary artery and PCV, although there was a tendency to a rise in the pulmonary arterial pressure during the infusion.

The systolic, diastolic and mean pressure in the brachial artery on the other hand showed a definite decrease. The fall in systolic pressure varied from 27 to 15 mm Hg, the diastolic from 31 to 15 mm Hg and the mean pressure from 24 to 10 mm Hg (Fig. 1, Table I).

*Cardiac output.*

The cardiac output at rest was in the two cases 10.2 and 11.5 l/min (mean values). During infusion of PGE (13 and 23 $\mu$g per min) the cardiac output decreased to 8.4 and 9.7 l per min respectively (Table I).

## Discussion.

From the present study it emerges that chemically pure PGE even in small amounts elicits rather marked and long-lasting actions in man. The most conspicuous effects on circulation was a fall in systolic and diastolic blood pressure and a rise in heart frequency. The latter effect may be secondary to the decrease in blood pressure since no effect was noted on the heart rate in the heart-lung preparation of the cat (Euler 1939). The cardiac output was slightly reduced.

While the systolic and mean systemic blood pressure fell considerably no such change was noted in the pulmonary arterial pressure. In spite of the fall in cardiac output the pressure in the right ventricle and the pulmonary arterial pressure were unchanged or slightly increased, indicating a rise in pulmonary vascular resistance. This effect is of interest in regard to the increase in resistance observed in the perfused cat's lung after prostaglandin (Euler 1939).

A peripheral vasodilatation was particularly noticeable in the face, but the available data do not indicate any gross change in total peripheral resistance.

Considering the small doses administered (0.2—0.7 μg/kg/min) the subjective symptoms were quite marked. The feeling of oppression in the head, followed by headache, was presumably caused by the vasodilatation in the head. Of the other symptoms the feeling of constriction in the pharynx and pressure in thorax were noteworthy.

The feelings of nausea and general discomfort at an infusion rate of 0.7 μg/kg/min prevented the use of larger doses.

The effects of PGE are as a rule fairly long-lasting, and this was also borne out by the present experiments. In addition, a certain degree of tachyphylaxis has been noted in tests on perfused organs (EULER 1939). In the present experiments a repeated higher dose had less effect on the recorded pressures and the heart rate, although the subjective symptoms were more marked.

## Summary.

1. Some effects of infusion of pure prostaglandin E (PGE) isolated from the vesical gland of sheep has been studied in two healthy subjects.

2. At infusion rates of about 0.2—0.7 μg/kg/min, PGE caused a fall in systolic and diastolic brachial artery pressure, and increased heart rate.

3. Cardiac output was moderately decreased.

4. No conspicuous changes were observed in right ventricle, pulmonary artery and PCV pressure.

5. Peripheral vasodilatation was observed in the face.

6. Subjective symptoms consisted in a feeling of warmth in the facial skin, headache, and, at higher infusion rates, general discomfort. Also constriction of the pharynx and thoracal oppression were noted.

7. Some of the effects persisted as long as 15 minutes after the end of infusions lasting 4—10 minutes.

A grant to one of us (S. B.) from the "Therese and Johan Andersson Memorial Foundation" is gratefully acknowledged.

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# A Spectrophotometric Method for Determination of Diamine Oxidase (DAO) Activity.

By

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Received 26 September 1958.

## Abstract.

HOLMSTEDT, B. and R. THAM. A spectrophotometric method for determination of diamine oxidase (DAO) activity. *Acta physiol. scand.* 1959. *45.* 152—163. — If diamine oxidase is allowed to act upon the diamine putrescine, a cyclic compound, $\Delta^1$-pyrroline, will be formed, which in turn reacts with o-aminobenzaldehyde to form a yellow compound. These reactions have been taken as a starting point for a photometric method for determination of diamine oxidase. If a solution containing o-aminobenzaldehyde, putrescine and a diamine oxidase preparation is incubated at 37° C a characteristic yellow colour is obtained. The intensity of the colour can be measured spectrophotometrically. The influence of pH and of the concentration of putrescine and o-aminobenzaldehyde on the reaction has been tested. The wavelength which gives a maximal extinction has been determined. The variation of the extinction with increasing time of incubation has been studied. On the basis of these investigations a standard procedure is suggested. Simultaneously determinations of diamine oxidase in different dilutions have been carried out using this colorimetric method and the Warburg technique.

Previous methods for estimation of diamine oxidase activity include determination of oxygen consumption (ZELLER and BIRKHÄUSER 1940), production of ammonia (ZELLER 1940) and production of hydrogen peroxide by a coupled reaction with indigodisulphonate (KAPELLER-ADLER 1956 a). The activity can also be estimated biologically (AHLMARK 1944) by the amount of substrate remaining after incubation with histamine.

In the general formula given by ZELLER (1951) for the oxidation of diamines

$$NH_2(CH_2)_nNH_2 + H_2O + O_2 \xrightarrow{DAO} NH_2(CH_2)_{n-1}CHO + NH_3 + H_2O_2$$

all resulting compounds have consequently been used for quantitative determination of the enzyme activity, save the aldehyde produced.

The method described in this paper has been based on the aldehyde formed when putrescine is oxidized by DAO. The putrescine aldehyde condenses to a cyclic compound, $\Delta^1$-pyrroline. This is allowed to react with o-aminobenzaldehyde and the resulting coloured compound, probably 2,3 trimethylene-1,2-dihydroquinazolinium hydroxide is measured in the Beckman spectrophotometer at 4,300 Å. The above mentioned substance has been synthetized by SCHÖPF (1936) and the enzymic formation of the same compound has been suggested by TABOR (1951).

## Experimental.

*Reagents used:* Ortho-aminobenzaldehyde, synthetized according to BAMBERGER and DEMUTH (1901, 1927). The compound was stored at — 8° C in ampoules containing nitrogen. Tetramethylene diamine dihydrochloride (Putrescine, Light & Co. Ltd. London). Aminoguanidine hydrocarbonate (Fluka A. G. Chemische Fabrik, Buchs SG). Semicarbazide hydrochloride (Merck, Darmstadt). Iproniazide (Hoffman La Roche, Basel). Trichloroacetic acid (TCA) 10—30 per cent. Phosphate buffer according to SÖRENSEN M/15, pH 6.8.

*Enzyme preparations:* Two sources of diamine oxidase were used:

1. Diamine oxidase (Nutritional Biochemicals Corporation, Ohio, USA).

2. A preparation from hog kidney according to a method described by ARVIDSSON, PERNOW, SWEDIN (1955). The description of these authors was followed in detail except for the final chromatographical separation, which was omitted. The enzyme was kept at — 8° C and was used as soon as possible because of the rapid decrease of the enzyme activity of the solution.

*Procedure:* The following dilutions were made up in test tubes for each determination:

1. 2.5 ml of o-aminobenzaldehyde (0.005 M, made up in phosphate buffer pH 6.8 and stable in solution at + 4° C for a week). Enzyme usually made up in phosphate buffer (2 to 20 mg enzyme powder per ml buffer). Phosphate buffer ad 4.5 ml.

2. 2.5 ml o-aminobenzaldehyde as above. Enzyme as above. Buffer ad 5 ml.

The test tubes were shaken at 37° C in a Warburg thermostat. After temperature equilibration 0.5 ml putrescine-hydrochloride (0.1 M freshly prepared in phosphate buffer) was added to test tube nr 1. In experiments with inhibitors these were dissolved in phosphate buffer. After the addition of o-aminobenzaldehyde, enzyme and inhibitor to the test tubes, these were placed in the thermostat for 2 hours before addition of substrate. This procedure was used in order to obtain equilibrium between enzyme and inhibitor before the addition of substrate. The usual duration of incubation after addition of substrates was three hours. At low enzyme activities longer incubation times were used. At the end of incubation 1 ml trichloroacetic acid (10 %) was added to both test tubes. (When the enzyme activity of tissue homogenates is tested it is advisable to use TCA of higher concentration). The tubes can then be kept for 24 hours. After centrifugation the extinction of the supernatant of test tube 1 was read in a Beckman spectrophotometer Model DU at 4,300 Å (length of cell 1 cm). The supernatant of test tube 2 was used as the blank.

When the extinction exceeded 0.8 the solution was diluted with aqua dest. in agreement with ordinary photometric technique. The extinction read was multiplied with the dilution.

The solutions mentioned under 1 and 2 were also used for Warburg determination of oxygen absorption at 37° C.

## Results.

*The extinction curve* for the yellow compound is presented in Fig. 1. Readings in the following experiments were always made at 4,300 Å. The absorption maximum remained the same whether or not TCA had been added for the precipitation of proteins.

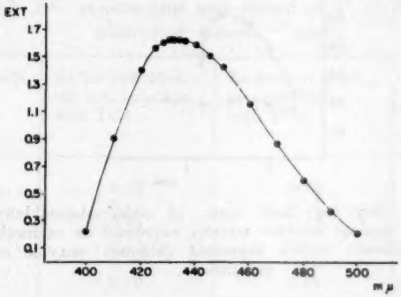



Fig. 1. Absorption spectrum of yellow compound (presumably 2,3-trimethylene-1,2-dihydroquinazolinium) formed when putrescine is incubated with DAO in the presence of o-aminobenzaldehyde.

*Activity pS-curve.* When the concentration of o-aminobenzaldehyde was kept constant at $2.5 \times 10^{-3}$ and the concentration of substrate varied (different enzyme activities being used) (Fig. 2) parabolic like curves resulted. The substrate optimum was found at a putrescine concentration of about $10^{-2}$ M. This concentration was used in all following experiments.

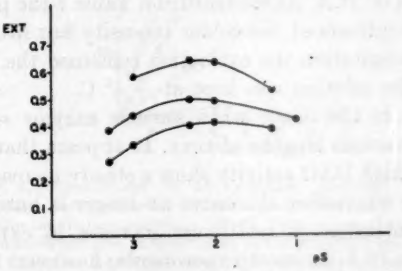



Fig. 2. Neg. log. mol. conc. of substrate plotted against the enzyme activity expressed as extinction. The different curves represent different enzyme concentrations.

*Optimum concentrations of o-aminobenzaldehyde* were obtained from the experiments demonstrated in Fig. 3. o-aminobenzaldehyde $2.5 \times 10^{-3}$ M was used throughout the investigation.

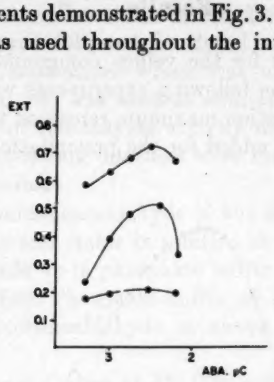



Fig. 3. Neg. log. mol. conc. of o-aminobenzaldehyde plotted against enzyme activity expressed as extinction. The different curves represent different enzyme concentrations.

*The pH optimum* of the reaction measured in a series of buffer solutions resulted in highest activity between pH 6.5—7.00. SÖRENSEN's phosphate buffer at pH 6.8 was therefore chosen for all subsequent experiments. The addition of putrescine, o-aminobenzaldehyde and enzyme to the buffer did not appreciably influence the pH. Even after 24 h incubation time no change above 0.1 pH was noticed.

It was found relatively impractical to read immediately large series of tests at an exact time of incubation. Therefore, the enzymic activity was interrupted by precipitation of the protein by the addition of TCA. As evident from Table I the precipitation of the proteins influenced the colour intensity but little. Furthermore, after precipitation the extinction remained the same for at least 24 h, if the solution was kept at + 4° C.

Fig. 4 presents the result when various enzyme solutions are incubated for various lengths of time. It appears that incubation solutions with high DAO activity show a steady increase in extinction up to 4 h whereafter the curve no longer is linear. At lower enzyme concentrations a rectilinear increase in extinction was obtained up to 12 h. In most experiments, however, the reaction was stopped at 3 h.

**Table I.**

*The effect on the extinction of precipitation of the proteins by the addition of TCA. Determinations with different amounts of enzyme. Volume of the incubation solution 10 ml instead of 5 ml as in the standard procedure. Final conc. of putrescine $10^{-3}$ M, of o-aminobenzaldehyde $2.5 \times 10^{-3}$ M. Time of incubation 3 h. At the end of the incubation time 1 ml of distilled water was added to 5 ml of the incubation solution and the absorption was measured. To the other part of the incubation solution 1 ml TCA (10 %) was added. The test tubes were centrifuged. The absorption of the supernatant was measured. The supernatant was stored at 4° C for 24 h and the absorption measured again.*

| mg Enzyme prep. per ml inc.-solution | Extinction without precipitation with TCA | Extinction after precipitation with TCA | Extinction 24 hours after precipitation with TCA |
|---|---|---|---|
| 0.1 | 0.09 | 0.09 | 0.10 |
| 0.2 | 0.18 | 0.16 | 0.17 |
| 0.4 | 0.36 | 0.30 | 0.30 |
| 0.6 | 0.50 | 0.45 | 0.45 |
| 0.8 | 0.63 | 0.59 | 0.59 |
| 1.0 | 0.79 | 0.67 | 0.68 |

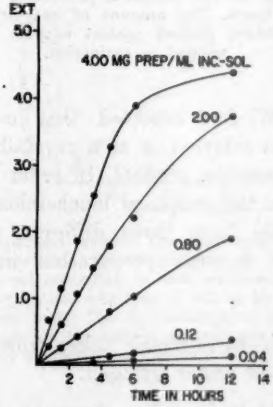



Fig. 4. Time-course of the reaction measured as extinction. Four different concentrations were used. Volume of the incubation solutions 20 ml. Final conc. of o-aminobenzaldehyde and putrescine as in the standard procedure. At different intervals after the addition of substrate aliquots of the incubation solution were taken up for measurement of extinction. Extinction plotted against time.

Under the above-mentioned optimal conditions a series of determinations with different enzyme concentrations were made. When the enzyme activity expressed as extinction was plotted against mg enzyme preparation per ml incubation solution a straight-line relationship was obtained (Fig. 5) allowing the estimation of various enzyme activities from the extinction.

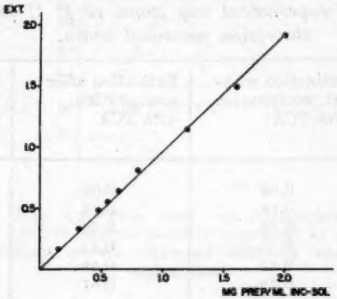



Fig. 5. Relationship between enzyme activity and extinction of incubation solution. Determinations carried out according to the standard procedure. Time of incubation 3 hours. The amount of enzyme per ml incubation solution plotted against enzyme activity expressed as extinction.

BAMBERGER (1927) has reported that o-aminobenzaldehyde when kept either in solution or as a crystalline substance polymerizes to a condensation product. In order to test the possible influence of this on the proposed biochemical reaction of DAO, o-aminobenzaldehyde from three different sources was tested against the same enzyme preparation in a series of solutions:

1. o-aminobenzaldehyde freshly taken from an ampoule where it had been kept under nitrogen.
2. o-aminobenzaldehyde taken from an ampoule that had been allowed to stand open for a month.
3. o-aminobenzaldehyde from a one week old solution kept at + 4° C in the refrigerator.

No significant differences in extinction were noted in experi-

ments using solutions from the three sources of o-aminobenzaldehyde. In the following experiments the solution of o-aminobenzaldehyde was prepared from the compound stored in ampoules with nitrogen and kept for no more than a week at + 4° C.

In order to make a comparison between the spectrophotometric method and the manometric method a series of experiments with different enzyme activities were performed where oxygen consumption and extinction measurements were made simultaneously. The reaction mixture mentioned under Experimental was brought into Warburg vessels and the oxygen consumption determined continuously with the development of the colour. The first reading of the Warburg manometer was taken 20 min after the addition of substrate and then at 15 min intervals up to 3 h. The rate of oxygen consumption was constant during this time. After this the incubation was terminated in the usual way by the addition of TCA. When the enzyme activity, expressed as extinction, measured after three hours was plotted against the same enzyme activity, expressed as oxygen consumption per hour and ml of incubation solution, (Fig. 6) a straight-line relationship was obtained.

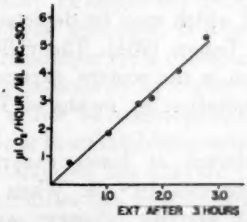
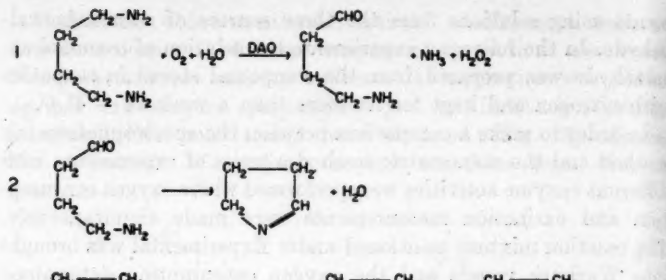



**Fig. 6. Comparison between the spectrophotometric and manometric methods. Determinations of DAO activity were carried out according to both methods parallel with the same incubation solutions (5 ml) as in the standard procedure (See text). The $O_2$-consumption per ml of the incubation solution per hour is plotted against the enzyme activity expressed as extinction after 3 hours.**

## Discussion.

According to the general formula given by ZELLER (1951) the biochemical reaction resulting in the yellow compound may be assumed to take place in the following way:



TABOR (1951) suggests that the aldehyde produced when putrescine is oxidized, is condensed to $\Delta^1$-pyrroline. This, in turn, reacts with o-aminobenzaldehyde to give a yellow compound, the formula of which may be depicted as above (SCHÖPF and OESCHLER 1936, TABOR 1951). The yellow colour obtained in the enzymic reaction in the writers' experience is stable for at least 24 h after precipitation by means of TCA of the proteins present.

The substrate optimum at lower enzyme activities using putrescine as substrate was $10^{-2}$ M. When enzymes of higher activity were used an activity pS-curve was obtained with a substrate optimum in the neighbourhood of $10^{-3}$ M. ZELLER (1939) measuring $O_2$ consumption using putrescine as substrate found a substrate optimum of $8 \times 10^{-3}$. The substrate optima of the manometric and the spectrophotometric methods thus are essentially the same. The shape of the activity pS-curve is also similar to that obtained by ZELLER in the substrate interval used by him.

The shape of the curve obtained when the reagent o-aminobenzaldehyde is used in increased concentrations may possibly be explained as a result of an inhibitory effect on DAO of this compound in excess.

The pH optimum for the development of the yellow colour in

the presented method falls within the same optimal interval as determined by BEST and MCHENRY (1930) with biological methods. It may, however, be mentioned that the formation of 2,3 trimethylene-1,2-dihydroquinazolinium in the spontaneous formation according to SCHÖPF and OESCHLER (1936) takes place at pH 5.

ZELLER (1939), SWEDIN (1943) and TABOR (1951) studied the time curve using oxygen consumption as a test of the oxidation of putrescine and histamine respectively. The rapidity with which oxygen was consumed decreased when one oxygen molecule per molecule substrate had been consumed. The rapidity of this second step of the reaction is considerably slower than that of the first one. It is assumed that an intermediate product of the substrate is formed. When ZELLER (1939) plotted activity pS-curves based on manometric measurements, he recorded the oxygen consumption only during the first 15 min after the addition of the substrate, where the second step of the reaction is negligible, especially if putrescine or cadaverine is used as substrate.

In the method presented in this paper the second step mentioned by ZELLER may be neglected because of the successive trapping of the aldehyde produced. It also appears from Fig. 5, that the reaction is rectilinear for a considerably longer time than 15 min. The decrease in activity at higher enzyme concentration may be attributed either to consumption of the substrate or o-aminobenzaldehyde or to an inhibitory action of 2,3 trimethylene 1,2-dihydroquinazolinium on the enzyme.

The first two possibilities can be calculated from Fig. 4 and 6. From Fig. 4 is seen that the decrease in enzyme activity occurs when the extinction of the incubation solution is 4. The amount of substrate and o-aminobenzaldehyde at the beginning of the reaction are respectively 10 $\mu$mole and 2.5 $\mu$mole per ml incubation solution. From Fig. 6 is seen that an enzyme activity which after 3 h of incubation has given an extinction of 2, is equivalent to the absorption of 3.6 $\mu$l $O_2$ per hour and ml incubation solution. This corresponds to 10.8 $\mu$l $O_2$ per 3 h and ml incubation solution and to $\frac{10.8}{22.4} = 0.48$ $\mu$mole $O_2$. If the reaction formula is correct, the same amounts of putrescine and o-aminobenzaldehyde, expressed as $\mu$mole, are consumed. Assuming a continuous straight line relationship of Fig. 6, the consumed amounts of either sub-

strate or o-aminobenzaldehyde equal 0.96 $\mu$mole per ml, when the extinction of the incubation solution is 4. Thus, about 90 (90.4) % of the substrate and 60 (61.6) % of the o-aminobenzaldehyde remains at the time when the reaction slows down (E = 4). The second possibility that the yellow compound inhibits the enzyme thus seems more justified. To confirm this 2,3 trimethylene 1,2-dihydroquinazolinium has to be made synthetically and compared to the yellow product resulting from the enzymic reaction. Such work is in progress.

Within a wide interval of enzyme activities a linear relationship exists with extinction, making it possible to use the developed method. To test the practicality of the method a series of inhibition curves has been performed with inhibitors of diamineoxidase. The results are presented in Fig. 7.

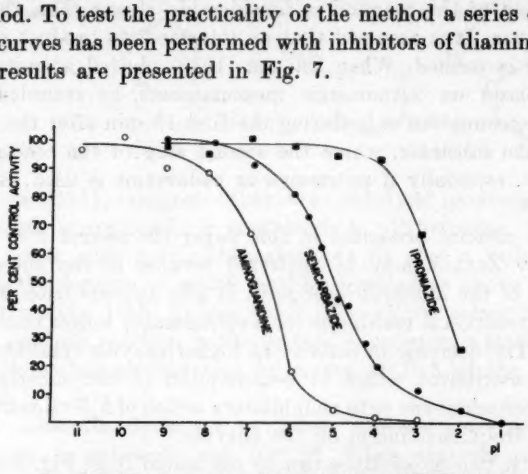



**Fig. 7.** Inhibition curves. Determinations of DAO activity were carried out according to the standard procedure. The amount of enzyme in the incubation solution was chosen to give an extinction of about 0.8 after 3 hours. The degree of inhibition is expressed in per cent activity of the inhibited enzyme compared with determinations with no inhibitor present.

pI = neg. log. mol. conc. of the inhibitor.

The sensitivity of the present method is lower than that of the biological method deviced by AHLMARK (1944) and modified by WIKSELL (1949). The biological method allows the determination of 0.005 $\mu$g histamine split per ml enzyme per hour. The present method expressed in the same units (unpublished experiments) would be equivalent to approximately 1 $\mu$g histamine per ml

per hour. Its sensitivity is about the same as that of the other chemical methods mentioned in the introduction. Detailed comparisons with other methods will follow in a subsequent paper. The method is simple to perform, requires little apparatus and many determinations can be made simultaneously by untrained personnel. It is suitable for biochemical work where for instance inhibition curves are desired, in which case the biological method is impossible to use. The enzyme activities of homogenates from various tissues can also be easily determined.

A method utilizing the trapping of the aldehyde according to the formula given has the advantage over the Warburg method that the influence of other oxidative processes and the further oxidation of the aldehyde are eliminated in the measurements.

In the past, doubts as to the homogeneity of diamine oxidase (histaminase) have been expressed (KAPELLER-ADLER 1956 b). If enzymes are defined according to their substrate specificity the present method in a true sense is a method for the determination of DAO, since it only works with diamines like putrescine and cadaverine.

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# Relaxation and Visco-Elastic Behaviour of Glycerol-extracted Muscle Fibre Bundles in the Presence of Zinc and ATP.

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Received 3 October 1958.

## Abstract.

EDMAN, K. A. PAUL. Relaxation and visco-elastic behaviour of glycerol-extracted muscle fibre bundles in the presence of zinc and ATP. *Acta physiol. scand.* 1959. *45.* 164—180. — Zinc-induced relaxation in glycerol-extracted rabbit psoas fibre bundles was studied under strictly isotonic and isometric conditions and compared with the effects of zinc on the visco-elastic behaviour of resting fibre bundles subjected to quick stretch. 0.2 mM zinc + 1 mM magnesium + 0.4 mM ATP does not affect the visco-elastic properties but induces relaxation of isometrically and isotonically contracted fibre bundles. Isotonic relaxation is complete only when the previous shortening does not exceed 25 percent of initial fibre length; the relaxing effect is practically absent when the bundle has shortened about 75 percent of its initial length. 2 mM zinc + 1 mM magnesium + 5 mM ATP and 4 mM EDTA + 4 mM magnesium + 4 mM ATP, which markedly decrease the visco-elastic resistance in the fibre bundle produces a pronounced isotonic relaxation even after extreme shortening of the bundles. Thus, the isotonic relaxation of glycerol-

extracted fibre bundles is dependent upon a decrease in the visco-elastic resistance. The isometric relaxation, on the other hand, does not need any pronounced visco-elastic change.

In an earlier publication (EDMAN 1958) certain effects of zinc on the isometric tension development of glycerol-extracted muscle fibre bundles were demonstrated. Among other effects it was shown that zinc (0.025—0.88 mM) in the presence of ATP[1] (0.4 mM) can reversibly relax isometrically contracted fibres. The effects of zinc are interesting considering that zinc is present physiologically in muscle tissue in concentrations as high as ~ 0.8 mmole/kg wet tissue. The present study extends the earlier work and treats the effects of zinc on the fibre bundles under strictly *isotonic* conditions. The work also includes recordings of the effect of zinc + ATP and EDTA + ATP upon the mechanical behaviour of resting fibre bundles subjected to stretch.

## Methods.

The glycerol extraction and preparation of the fibre bundles from rabbit psoas were carried out as described earlier (EDMAN 1957). A KCl solution with pH 7.30 containing 10 mM diethylbarbituric acid (Veronal), 100 mM K and 1 mM Mg was used as medium after the glycerol extraction. Hereafter this solution will be called "veronal buffer". Ethylenediamine tetraacetate, disodium dihydrate, pro analysi, E. Merck, was used. For data on the quality of the other chemicals used, see EDMAN (1958).

In addition to the dibarium salt of ATP, Pabst lot no. 129 (EDMAN 1958), the disodium salt of ATP, "chromatographically pure", Pabst lot no. 109 A, was used. According to an analysis performed with the technique described earlier (EDMAN 1957), the amount of total nucleotide in 37.4 mg of the $Na_2H_2ATP$ substance was 0.0581 mmole. The relative nucleotide concentrations were: ATP 96 %, ADP 2 %, AMP < 0.5 % and an unknown fraction, possibly uridine nucleotide, < 1 %. The solution was prepared by dissolving the $Na_2H_2ATP$ salt in the veronal buffer and correcting the pH to 7.3 with KOH.

The water employed for washing and for preparation of solutions was double distilled in borosilicate glass. The pH determinations were done with a glass electrode. The experiments were performed at room

[1] The following abbreviations will be used: ADP, adenosinediphosphate; AMP, adenosinemonophosphate; ATP, adenosinetriphosphate; EDTA, ethylenediamine tetraacetate.

temperature, 20—22° C. If not otherwise stated the data on the concentrations refer to the total concentration in the bath.

The veronal buffer solution used as medium always contained 1 mM Mg (see above). Therefore, the Mg concentration will not be specially mentioned in the rest of the communication unless the concentration was different from usual.

*Isotonic recording.*

Two identical devices for isotonic recording with front-writing pens were used in parallel. Before use the pens were completely balanced in air. To achieve this it was necessary that the front-writing part be separately completely balanced. The tip of the writing part was a glass bead and was applied to the smoked drum by light pressure from a fine coil spring mounted around the axis of the writing part. (With conventional technique the tip is pressed against the paper by gravity which presupposes that the centre of gravity of the writing part does not fall on its axis.) The tempered and polished conical steel tips comprising the ends of the main axis were mounted in jewelled bearings. The kymograph paper was lightly sooted. To further reduce the friction the kymograph was kept faintly vibrating by an a.c.magnet. The pen was applied to the drum in a manner such that the rotation of the main axis was approximately equally distributed above and below the horizontal line during an ordinary contraction. Within the recording range used in the present investigation the tension of the bundle varied less than 1 mg. Shortening of the fibre bundle from 100 % to 75 % of the initial length (40 mm) corresponded to 19.1 mm on the kymogram, shortening from 75 % to 50 % of the initial length to 19.1 mm and shortening from 50 % to 25 % to 19.6 mm. The mean value (19.3 mm) will be used hereafter for 10 mm contraction of the fibre bundle regardless of the degree of contraction.

A loop of platinum wire (0.15 mm in diameter) with a weight of 7.5 mg was attached to the end of the fibre bundle that would become the lower end after mounting; a nylon thread (0.08 mm in diameter) was attached to the other end. The length of the fibre bundle between the attachments of the platinum loop and the nylon thread was 40.0 mm; for data on the thickness of the bundles, see later. During the experiments the fibre bundles were mounted vertically between a definite point on the pen and a platinum hook fused into a glass rod. The bath solution (40 ml) was kept in a cylindrical glass vessel. The exchange of the bath solution was carried out by exchanging the glass vessel for another containing the new solution. In this procedure the vessels were moved vertically, *i. e.* in the longitudinal direction of the fibre bundles. The bundles were not exposed to air for more than 10 sec. during the mounting to the pens and 4—5 sec. at most during the exchange of solutions.

The *shortening* experiments were carried out using 0.4 mM ATP and a load of 9 mg. Before the start of the experiments the fibre bundles lay in a Petri dish containing 60 ml ATP-free test solution

(0.01 mM zinc) or, respectively, control solution (veronal buffer only) for 1—2 hours. Cross section of the fibre bundles was 103—150 × 178—299 $\mu$.

*Relaxation* was induced with 0.2 mM zinc + 0.4 mM ATP at a load varied between 59 and 78 mg in the different experiments ($T_2$ in Fig. 2). The fibre bundle was first contracted with 0.4 mM or 0.8 mM ATP at a load of 9 or 24 mg ($T_1$ in fig. 2). When the desired degree of shortening had been attained, the load was increased to $T_2$ and the ATP concentration was changed to 0.4 mM if a higher concentration of ATP had been used to induce the contraction. The relaxation was not started until a constant length had been reached under the new condition. From 4 to 8 contraction-relaxation cycles were carried out with each fibre bundle. During the different cycles the fibre bundles were allowed to shorten to varying degrees before relaxation was induced. The relaxation was recorded until constant length was attained, and the fibre bundle was subsequently washed with buffer solution for about 30 min, whereafter a new contraction was started.

In some experiments the relaxation was induced with 2 mM zinc + 5 mM ATP. The fibre bundle in these experiments was contracted by 0.8 mM ATP at a load of 23 mg. When the desired degree of shortening had been attained, the bundle was pretreated with 0.07 mM zinc alone for 2—3 min. at a load of 68 mg. The cross section of the fibre bundles used was: 94—168 × 206—262 $\mu$.

Because of the complex formation between ATP and metals it is necessary to increase the total zinc concentration as well when the concentration of ATP is raised in order to keep the zinc ion concentration sufficiently high. Assuming a stability constant (log K) of 4.00 for the Mg-ATP complex (MARTELL and SCHWARZENBACH 1956: 4.00; RAAFLAUB 1956: 4.00; WALAAS 1957, 1958: 4.04; G. WEIZEL, pers. comm.: 4.04) and 4.76 for the zinc-ATP complex (G. WEIZEL, personal communication) the combination 0.2 mM zinc + 0.4 mM ATP corresponds to 0.071 mM $Zn^{++}$ and 0.032 mM free ATP. The combination 2 mM zinc + 5 mM ATP corresponds to 0.017 mM $Zn^{++}$ and 2.1 mM free ATP. The concentration of 2 mM total zinc was arrived at by experiment and is close to the lower limit giving complete isometric relaxation in the presence of 5 mM total ATP (EDMAN, unpublished).

*Isometric recording.*

The same technique was used as that described in a previous paper (EDMAN 1957).

*Visco-elasticity measurements.*

The principal aspects of the technique in measuring the elasticity have been described earlier (EDMAN 1957, page 236). The cross section of the fibre bundles used was 84—131 × 164—229 $\mu$ and the length 10.0 mm. A quick stretch was performed in 2 sec. The stretch comprised 0.83 % of the fibre length in the control experiments (veronal buffer alone) and in the experiments with zinc; in the experiments

with EDTA the fibre bundles were stretched by 2.5 % of their initial length because of the greater elastic compliance in these experiments. The force necessary to stretch the fibre bundle was recorded on the smoked drum. The recording was continued until equilibrium tension had been attained. In the control experiments and in the experiments with 0.2 mM zinc + 0.4 mM ATP the tension was always recorded during 5 min. after stretch.

The fibre bundles were given an initial tension of 22.2 mg while still in the veronal buffer. In the *control experiments* (veronal buffer alone) the stretch was carried out soon after the fibre bundle had been put into position in the recording device. In the experiments with *0.2 mM zinc + 0.4 mM ATP* an isometric contraction-relaxation cycle was produced before the visco-elasticity measurement. The fibre bundles in these experiments were contracted isometrically with 0.4 mM ATP for two min. and after that washed with buffer solution alone for two min. and for an additional 5-minutes with 0.2 mM zinc. Relaxation was then induced with 0.2 mM zinc + 0.4 mM ATP and the tension was recorded for 5 min. when it was certain that equilibrium had been attained. After the fibre bundle had been immersed in the solution of 0.2 mM zinc + 0.4 mM ATP for 60 min. the stretch was performed. In the experiments with *2 mM zinc + 5 mM ATP* and with *4 mM EDTA + 4 mM Mg + 4 mM ATP* the test solution was applied without previous contraction of the bundle. Immediately after the immersion in the test solution an increase in tension occurred initially and subsided completely in the experiments both with EDTA and with zinc. The stretch was carried out in these experiments after the fibre bundle had remained in the test solution for 10 min. when constant tension had certainly been reached.

The statistical significance of the results has been calculated using Fisher's t-test.

## Results.

### *1. Effect of 0.01 mM zinc on isotonic contraction.*

Fig. 1 shows the effect of 0.01 mM zinc on isotonic contraction of glycerol-extracted fibre bundles at 0.4 mM ATP concentration. Not unexpectedly the effect is similar to that described in isometric contraction (EDMAN 1958), *i. e.* an inhibition during the entire course of the shortening. This is clearly evident from Table I, in which the results obtained in this study on isotonic contraction are presented together with the earlier results obtained in studies on the effect of 0.01 mM zinc and 0.22 mM ATP on isometric contraction for the purpose of comparison. The initial absolute and relative rates of contraction in isotonic contraction are defined in analogy with the definition given for isometric recording (EDMAN

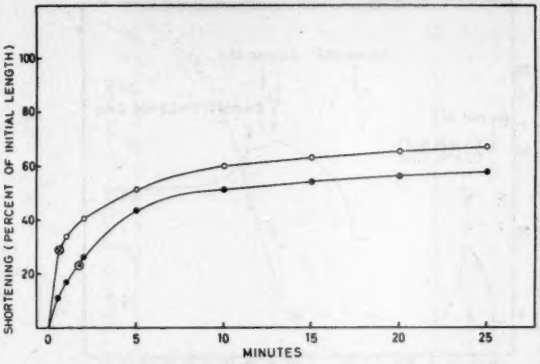


Fig. 1. Effect of 0.01 mM zinc on the isotonic contraction of glycerol-extracted fibre bundles. ATP concentration 0.4 mM. Load 9 mg. Cross section of the fibre bundles: 103—150 × 178—299 $\mu$. Filled circles: test experiments, open circles: control experiments, both representing the mean shortening after different time intervals. 4 experiments in both groups. The average time for reaching 40 % of the final degree of shortening is indicated with ⊗ for the control curve and with ⊙ for the test curve. The standard error of the means does not exceed the size of the symbols.

1957, 1958). The initial *absolute* rate in an isotonic contraction is thus the mean shortening per unit of time during the time interval from 0 to 40 % of the magnitude of the final contraction. The initial *relative* contraction rate is the reciprocal of the time required to attain 40 % of the final level of the contraction. The

**Table I.**

*Effect of 0.01 mM $ZnCl_2$ on the isotonic and isometric contraction of glycerol-extracted muscle fibre bundles in the presence of 1 mM magnesium.*

| Mode of contraction | Initial relative rate | Initial absolute rate | Final tension | Number of pairs of expts. |
|---|---|---|---|---|
| Isotonic ..... | —66*** | —71*** | —14** | 4 |
| Isometric ..... | —81*** | —82*** | — 9* | 17 |

Isotonic contraction induced by 0.4 mM ATP, isometric contraction by 0.22 mM ATP. The effect is given as the difference between the mean of the tests and the mean of the controls in percentage of the controls.

*** = $P < 0.001$, ** = $0.001 < P < 0.01$, * = $0.01 < P < 0.05$.

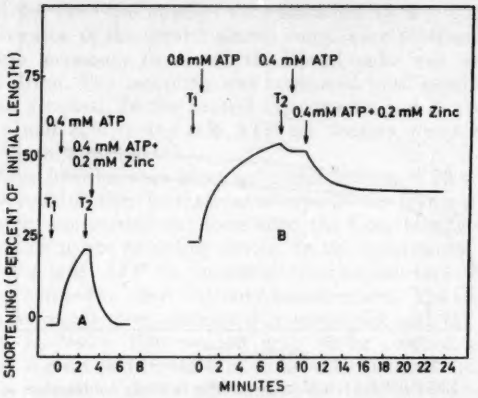


Fig. 2. Repeated isotonic relaxations with 0.2 mM zinc + 0.4 mM ATP in a glycerol-extracted fibre bundle at different degrees of shortening. Two contraction-relaxation cycles preceded the first cycle (A) shown in the figure. Between A and B three contraction-relaxation cycles were performed. After each relaxation the bundle was washed for 30 min. with zinc-free buffer solution. At $T_1$ the bundle was loaded with 24 mg, at $T_2$ with 72 mg. Cross section of the fibre bundle: 131 × 206 $\mu$. Note the slower and smaller relaxation at the higher degree of shortening.

isotonic contraction is slow and is not entirely completed even after one hour (EDMAN 1953). The contraction level 25 minutes after the start was selected as an approximate value for the final level in this study.

*2. Isotonic relaxation by zinc in the presence of ATP.*

In one series of experiments isotonic relaxation from different degrees of shortening was induced with 0.2 mM zinc + 0.4 mM ATP. Several contraction-relaxation cycles were performed with each fibre bundle (see Methods). A typical example is presented in Fig. 2. The purpose of the examination was to study the relaxation ability at various degrees of deformation of the contractile protein. The lengthening has therefore been calculated in percentage of the actual total shortening consisting of the newly-induced shortening and the remainder of the previous contraction, if this had not been completely relaxed. The results are summarized in Fig. 3. It may be seen that complete relaxation was

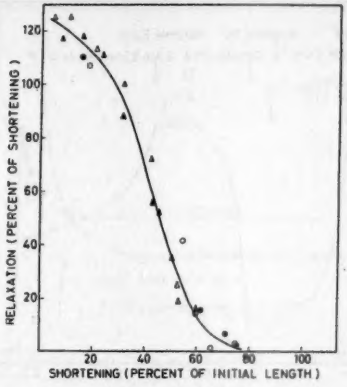


Fig. 3. Isotonic relaxation of glycerol-extracted fibre bundles with 0.2 mM zinc + 0.4 mM ATP in relation to the degree of shortening. Abscissa: degree of shortening when relaxation is induced, in percentage of the initial length of the fibre bundle. Ordinate: relaxation as percentage of the previous shortening. Cross section of the fibre bundles:

△ 131 × 206 μ, ▲ 94 × 197 μ,
○ 140 × 243 μ, ● 122 × 243 μ.

obtained, *i. e.* the fibre bundle stretched to at least its initial length, if it had not shortened by more than about 25 % of its initial length. At lower degrees of shortening the relaxed length even exceeded the initial length. When the shortening was continued to more than 25 % of the initial length, the capacity for relaxation decreased; when the fibre bundle shortened by 75 % of its initial length, no relaxation was obtained. The results are not markedly modified by the presence of a contraction remainder from an earlier contraction-relaxation cycle.

With 2 mM zinc + 5 mM ATP a pronounced relaxation can be induced (about 50—100 % of the shortening) at the load used here even if the fibre bundle has been allowed to shorten by about 60 % of its initial length as shown in Fig. 4.

In the presence of high concentrations of ATP (4—6 mM) the Marsh-Bendall factor (BENDALL 1953) and EDTA (WATANABE and SLEATOR 1957) are also able to produce a total or almost total isotonic relaxation at about 60 % shortening of the fibre

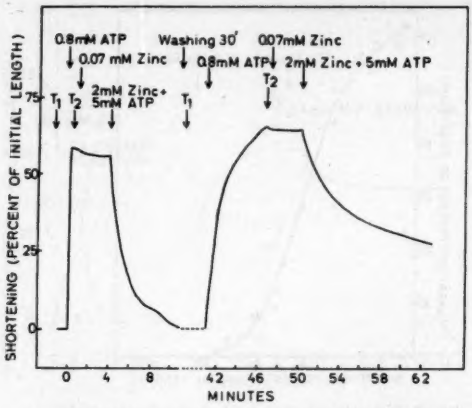


Fig. 4. Isotonic relaxation of a glycerol-extracted fibre bundle with 2 mM zinc + 5 mM ATP. At $T_1$ the bundle was loaded with 23 mg, at $T_2$ with 68 mg. Washing after the first relaxation performed with veronal buffer solution alone. Cross section of the fibre bundle: $108 \times 224\ \mu$.

bundle. The possibility of producing complete relaxation of a 60 % shortened fibre bundle with 4 mM EDTA + 4 mM Mg + 4 mM ATP as described in the report of WATANABE and SLEATOR (1957) has been verified under the experimental conditions used in the present study.

Since ATP has only a limited depth of penetration into glycerol-extracted fibres and fibre bundles (see Discussion, EDMAN 1957), the central parts will remain rigid and will consequently be passively deformed during isotonic contraction. In order to determine if the geometric aspects of the fibre bundle as a whole are changed thereby, the fibre bundle was subjected to direct microscopic inspection while in the vessel of bath solution during the course of isotonic contraction and relaxation in several experiments (magnification 6—40 ×). It was found that the fibre bundles retained parallelism of the visible fibres even under extreme shortening. No changes in the geometry of the fibre bundle, *e. g.* twisting or folding, which might explain why the capacity for relaxation with zinc + low ATP concentration diminishes with high-grade shortening could be demonstrated. The results are further discussed later.

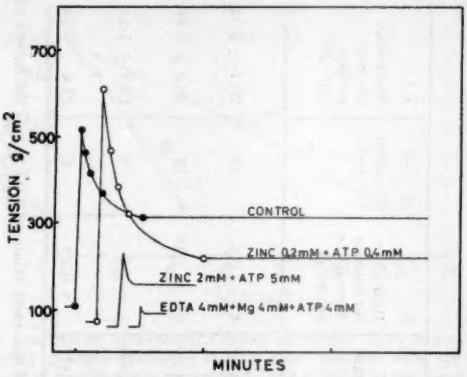


**Fig. 5. The behaviour of glycerol-extracted fibre bundles at and following stretch. Stretch, comprising 0.83 % of the fibre length in the controls and in the experiments with zinc and 2.5 % of the fibre length in the experiments with EDTA, was achieved in 2 sec. The control curve (veronal buffer alone) and the curve for 0.2 mM zinc + 0.4 mM ATP are derived from the means of the experiments presented in Table II for the respective group. At the last symbols in these curves equilibrium tension has been reached (tension recorded 5 min). The curves for 2 mM zinc + 5 mM ATP and for EDTA are typical examples of experiments in these groups. The curves are arbitrarily displaced along the abscissa.**

*3. Visco-elastic behaviour of glycerol-extracted muscle fibre bundles.*

When a fibre bundle is quickly stretched there is an initial steep rise in tension ("initial response") followed by a decrease until an equilibrium tension has been reached. The reciprocal of the initial rise in tension is an arbitrary measure of the elastic compliance[1] of the fibre bundle, while the subsequent decrease in tension is due to *visco*-elasticity. Table II presents quantitative data from the recordings and Fig. 5 shows the typical forms of the curves obtained.

The combination 0.2 mM zinc + 0.4 mM ATP does not significantly change the initial response to stretch and the subsequent

[1] The compliance calculated in this way is a "dynamic elasticity". PORTZEHL (1952), later quoted by WEBER and PORTZEHL (1954) and WEBER (1955, 1958), calculates the modulus of elasticity from the tension obtained 2 minutes after stretch, thereby obtaining a measure of the "static elasticity". In PORTZEHL's experiments the stretching comprised 5—10 % of the fibre length.

**Table II.**

*Effects of zinc + ATP and EDTA + ATP on the visco-elastic behaviour of glycerol-extracted muscle fibre bundles at and following stretch.*

| Test medium | 1 Equilibrium tension of fibre bundle *before* stretch g/cm² | 2 Stretch in % of initial fibre length | 3 Initial tension increase at stretch g/cm²/% stretch | 4 Equilibrium tension increase after stretch g/cm²/% stretch | 5 Half time for tension decrease in seconds | 6 Number of expts. | 7 Mean area of cross section $cm^2 \cdot 10^5$ |
|---|---|---|---|---|---|---|---|
| Veronal buffer alone (1 mM Mg)..... | 108 ± 3 | 0.83 | 506 ± 39 | 259 ± 36 | 4.1 ± 0.3 | 13 | 20.7 ± 0.52 |
| Zinc 0.2 mM + Mg 1 mM + ATP 0.4 mM ..... | 73 ± 7*** | 0.83 | 642 ± 33* | 188 ± 12 | 6.3 ± 1.0* | 13 | 20.0 ± 0.91 |
| Zinc 2 mM + Mg 1 mM + ATP 5 mM ..... | 76 ± 11* | 0.83 | 187 ± 12*** | 52 ± 18*** | ~ 1[a] | 6 | 19.8 ± 1.25 |
| EDTA 4 mM + Mg 4 mM + ATP 4 mM ..... | 65 ± 11** | 2.5 | 20 ± 2*** | 15 ± 2*** | ~ 0.5[a] | 6 | 21.6 ± 0.35 |

The degree of significance has been calculated for the differences between the means of the tests (zinc or EDTA) and the means of the controls (veronal buffer alone). Asterisks: see Table I.

a. No statistical calculation has been performed but the values were quite outside the range of the controls

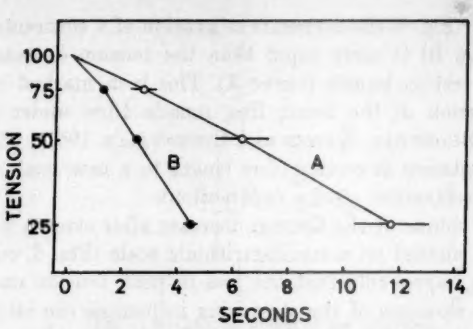


Fig. 6. Comparison between the decay of tension after stretch (curve A) of resting glycerol-extracted muscle fibre bundles and the relaxation (curve B) of isometrically contracted fibre bundles in the presence of 0.2 mM zinc + 0.4 mM ATP. Abscissa: time in sec. after the start of the tension decrease. Ordinate: tension remaining in excess of the equilibrium tension, expressed in percentage of total tension decrease. Relaxation and stretch experiments performed in succession on the same fibre bundle. Number of bundles: 13. The stretch experiments are the same as those shown in Table II for 0.2 mM zinc + 0.4 mM ATP.

The standard error of the means is indicated with a horizontal bar. Ratios between the times for tension decrease after stretch and relaxation:

At 75 %: $2.2 \pm 0.35$, $P < 0.01$;
at 50 %: $2.6 \pm 0.39$, $P < 0.01$;
at 25 %: $2.7 \pm 0.36$, $P < 0.001$.

decrease in tension. The combination 2 mM zinc + 5 mM ATP, on the other hand, increases the elastic compliance; the initial response to stretch is decreased 2.7 times. Furthermore, there is a more rapid decline in tension after the initial response, and, the equilibrium tension increase after stretch (= the equilibrium tension after stretch minus the initial tension before stretch) is 5 times smaller. With 4 mM EDTA + 4 mM Mg + 4 mM ATP the initial response to stretch is reduced 25 times and the equilibrium tension is attained almost instantaneously. It is conceivable that some yielding may already occur during stretch, especially in the case of EDTA + ATP, where the tension decrease is very rapid. This would cause an underestimation of the initial response to stretch.

In the experiments with 0.2 mM zinc + 0.4 mM ATP the relaxing effect and the effect on the visco-elastic behaviour at and following stretch were studied in the same fibre bundle. As is

evident from Fig. 6 the isometric relaxation of a contracted fibre bundle (curve B) is more rapid than the tension decrease after stretch of a resting bundle (curve A). This is in marked contrast to the situation in the living frog muscle fibre under isotonic conditions (BUCHTHAL, KAISER and ROSENFALCK 1951), where the passive adjustment of resting fibre length to a new load is much faster than relaxation after a contraction.

The time course of the tension decrease after stretch is almost linear when plotted on a semilogarithmic scale (Fig. 6, curve A). Hence, it is improbable that the loss in peak tension caused by the relative slowness of the stretching influences the estimate of the rate of tension decrease. The rate of relaxation (curve B) may be limited by the rate of diffusion of ATP into the fibre bundle. This will cause an initial delay in relaxation and an underestimation of the difference between the rate of tension decrease after stretch and the rate of relaxation. It is therefore probable that the differences between the courses of relaxation and tension decrease after stretch obtained with whole fibre bundles are really due to differences in the events within the single contractile units at relaxation and following stretch. The results are interpreted below.

## Discussion.

### *1. Contraction.*

Because of the passive resistance of the ATP-free central parts of the fibre bundle and of the individual fibres during shortening the degree of shortening will be determined not only by the contractility of the individual elements but also by the thickness of the peripheral, active layer.

The effects of 0.01 mM zinc on the ATP-induced contraction of glycerol-extracted fibre bundles are very similar in isotonic and isometric recording. The final level is less inhibited by zinc than the initial phase of the contraction. The explanation given for the isometric case (EDMAN 1958) is probably also applicable to the isotonic one.

### *2. Relaxation.*

The visco-elastic properties of a muscle fibre bundle are complicated. A comprehensive treatment of these problems is given by BUCHTHAL *et al.* (1951) and REICHEL (1952). As a first ap-

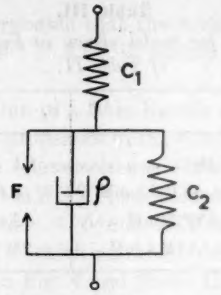


Fig. 7. Mechanical model of the glycerol-extracted muscle fibre bundle. $C_1$ and $C_2$ elastic elements, $\varrho$ viscous element and F force (= active contractility).

proximation the passive properties of the fibre bundle can be described with the model in Fig. 7. An elastic element with compliance $C_1$ is in series with another elastic element with compliance $C_2$, which is shunted by a viscous element with fluidity $\varrho$ symbolized by a dash pot. In the general case of a visco-elastic body a series dash pot is also present, but, since the fibre bundle can carry a load indefinitely, no such element can be present in our case. The active contractility can be represented by a force F, which acts in parallel with $C_2$ and $\varrho$. According to BUCHTHAL *et al.* (1951) it is certainly more adequate to use a system composed of several of the units described here coupled in series. However, the single unit may be used for the following simple considerations.

Using this model, it follows that the initial tension increase after stretch is proportional to $1/C_1$, that the equilibrium tension increase is proportional $1/(C_1 + C_2)$, and, that the half-time of the system is proportional to $[1/(1/C_1 + 1/C_2)] \cdot 1/\varrho$.

From the measurements reported in Table II, it is consequently possible to calculate the changes in $C_1$, $C_2$ and $\varrho$ caused by the various treatments. These are shown in Table III.

Taking the experimental uncertainties illustrated in Table II into account, it appears from Table III that 0.2 mM zinc + 0.4 mM ATP does not significantly affect the visco-elastic properties of the fibre bundle. With 2 mM zinc + 5 mM ATP the compliances

**Table III.**

*Visco-elastic parameters for model shown in Fig. 7 calculated from data of Table II.*

| Test solution | $C_1$ | $C_2$ | $\varrho$ |
|---|---|---|---|
| Veronal buffer alone (1 mM Mg) ..... | 1 | 1 | 1 |
| Zinc 0.2 mM + Mg 1 mM + ATP 0.4 mM . | 0.79 | 2.0 | 0.74 |
| Zinc 2 mM + Mg 1 mM + ATP 5 mM .... | 2.7 | 7.4 | ~ 17 |
| EDTA 4 mM + Mg 4 mM + ATP 4 mM .. | 25 | 8.4 | ~100 |

$C_1$ and $C_2$ both increase, as does the fluidity $\varrho$. Similar but even more pronounced effects are found with 4 mM EDTA + 4 mM Mg + 4 mM ATP.

The isometric tension of a fibre bundle can be lowered either by an unspecific increase in $C_1$ or by a real relaxation, *i. e.* by disappearance of the force F and return to rest length of the contractile elements.

The fact that reversible, isometric relaxation can be obtained with 0.2 mM zinc + 0.4 mM ATP without any significant change in $C_1$ makes it seem probable that the effect of the treatment with zinc + ATP is a real relaxation, according to the definition given above.

To what extent is a certain fluidity ($\varrho$) needed for relaxation? In a contraction that is strictly isometric even at the submicroscopic level, no fluidity would be necessary for relaxation, which would be a simple disappearance of a force. If, on the other hand, a series elastic component is present, there is also a shortening of the contractile unit, and it is probable that the disappearance of this shortening needs some fluidity. The need for fluidity should be greatest in relaxation from maximal isotonic shortening.

If isometric contraction of a fibre bundle includes shortening of the contractile units, the rate of relaxation could be dependent upon visco-elastic factors. However, if the visco-elastic factors were rate-determining, the isometric relaxation with 0.2 mM zinc + 0.4 mM ATP should follow the same time course as the tension decrease after stretch when the bundle has been equilibrated with the same solution. This is not the case as shown in Fig. 6. Moreover, no definite change in passive visco-elastic properties could be demonstrated with 0.2 mM zinc + 0.4 mM ATP. From these

facts it seems most probable that the reversible *isometric* relaxation produced by zinc + ATP is mainly a disappearance of a force.

The *isotonic* relaxation of a fibre bundle seems to be dependent upon the visco-elastic properties of the bundle. In extremely shortened fibre bundles no relaxation was obtained, at the load used, with 0.2 mM zinc + 0.4 mM ATP, but complete relaxation with the combinations 2 mM zinc + 5 mM ATP and 4 mM EDTA + 4 mM Mg + 4 mM ATP which markedly decrease the visco-elastic resistance, as shown in Fig. 5 and Table III. The role of the passively deformed, uncontracted parts of the fibre bundle during the isotonic relaxation is unclear. If the inactive parts have been plastically deformed during the shortening, it is conceivable that they could hamper the relaxation of the bundle. On the other hand, if they have been elastically deformed, they could be expected to facilitate the lengthening of the bundle during the relaxation.

## Summary.

1. Zinc in 0.01 mM total bath concentration brings about partial inhibition of the isotonic contraction of glycerol-extracted rabbit psoas fibre bundles induced by 0.4 mM ATP in a manner similar to that found in isometric contraction.

2. Isotonically contracted fibre bundles loaded with about 250 g/cm² relax upon addition of 0.2 mM zinc + 1 mM Mg + 0.4 mM ATP. The relaxation is complete when the previous shortening does not exceed 25 % of initial fibre length; the relaxing effect decreases with an increase in the degree of previous shortening and is practically absent when the bundle has shortened about 75 % of its initial length. With 2 mM zinc + 1 mM Mg + 5 mM ATP it is possible to produce a pronounced relaxation even after extreme shortening as is also possible with 4 mM EDTA + 4 mM Mg + 4 mM ATP.

3. The effect of zinc + ATP and EDTA + ATP on the visco-elastic behaviour at and following stretch of resting fibre bundles was studied. No effect could be demonstrated with the combination 0.2 mM zinc + 1 mM Mg + 0.4 mM ATP, which is able to bring about a rapid isometric relaxation of contracted fibre bundles. The combination 2 mM zinc + 1 mM Mg + 5 mM ATP, on the other hand, increased the compliance of the elastic

components and, in addition, decreased the viscous resistance of the contractile unit. Similar, but more pronounced effects were obtained with the combination 4 mM EDTA + 4 mM Mg + 4 mM ATP.

4. In the discussion it is pointed out that the effect of zinc + ATP is a real relaxation, *i. e.* a disappearance of the force and a return to rest length of the contractile elements. Disappearance of the force, probably independent of changes in visco-elastic properties, seems to be the most important factor in *isometric* relaxation of glycerol-extracted muscle fibre bundles.

This investigation was supported by grants from the Medical Faculty of the University of Uppsala and the Magnus Bergvall Foundation.

I wish to thank Professor G. Weitzel for kindly supplying unpublished stability constants of metal-ATP complexes and Professor Ernst Bárány for his helpful criticism and stimulating interest in this investigation.

The technical assistance of Mrs. Birgit Andersson is gratefully acknowledged.

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Acta physiol. scand. 1959. 45. 181—189.

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# Serum Lipid Composition in Experimental Hypercholesterolaemic Rats Given Polyenoic Fatty Acids.

By

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Received 10 October 1958.

## Abstract.

HAUGE, JENS G. Serum lipid composition in experimental hypercholesterolaemic rats given polyenoic fatty acids. *Acta physiol. scand.* 1959. *45*. 181—189. — It has previously been established that 20—100 mg of polyunsaturated oils given daily to young rats raised on a 1 % cholesterol—10 % hydrogenated coconut oil diet results in a pronounced reduction of the serum cholesterol concentration. By means of extraction of the serum lipids, chromatographic separation, and alkali isomerization it has now been shown that this decrease in serum lipids, reflected in reductions in both free and esterified cholesterol and in non-cholesterol lipids, is accompanied by characteristic changes in the polyenoic fatty acid composition of the cholesterol esters. Linoleate supplementation resulted in the appearance of more dienoic and tetraenoic acid in the esters, linolenate particularly in the appearance of pentaenoic, but also some hexaenoic acid. Cod liver oil had an effect on all polyenoic constituents, and the magnitude of this effect, referred to the actual content of such acids in the oil, was by far the strongest. This indicates a preference in the organism for the ready

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made polyunsaturated acids. The largest reductions in total cholesterol were correlated with a preponderance of linolenic acid-type unsaturation in the esters, a fact which emphasizes the relative inferiority of linoleic acid in the aspect of cholesterol metabolism here studied.

In previous papers from this laboratory (HAUGE and NICOLAYSEN 1958 b, c) it was reported that 20—100 mg of polyenoic fatty acids given daily for 14 days to rats raised on a 1 % cholesterol diet containing 10 % hydrogenated coconut oil as the only source of fat, resulted in a pronounced depression in the total serum cholesterol, as well as in liver cholesterol. The present paper reports experiments investigating this effect on the serum lipids in further detail, especially with respect to the fatty acid composition of the cholesterol ester fraction.

## Experimental.

*Biological.* The animals were the same as those used for series I in HAUGE and NICOLAYSEN (1958 c). Equal individual aliqots of blood sera from the 14th day of supplementation were pooled for each of the 9 groups, the number of animals per group ranging from 10 to 14. Five rats from our stock colony were also sacrificed, and the combined blood serum prepared.

*Chemical methods.* Total serum lipids were extracted with methylal-methanol (4 : 1), a solvent mixture used for this purpose by FILLERUP and MEAD (1953). In our hands, the subsequent evaporation of the solvent to dryness and reextraction of the lipid with pet. ether resulted in grave losses. The following procedure was therefore employed: 6 ml serum was stirred with 90 ml methylal-methanol for 30 minutes at about 40° C. The suspension was filtered, and a little more than half the solvent evaporated, sufficient to make the lipids partly insoluble. The emulsion was extracted twice with equal volumes of pet. ether (B. P. 40—60° C), and the pet. ether phase dried over anhydrous $Na_2SO_4$. It was then reduced in volume and transferred to a weighed tube, the remaining solvent thereafter removed with $O_2$-free $N_2$. After weighing, the total lipid was dissolved in 2 ml pet. ether, 2 aliqots of 5 $\mu$l taken for cholesterol determination (HAUGE and NICOLAYSEN 1958 a), and the remainder stored under $N_2$ in the cold until chromatography. The completeness of this extraction procedure was satisfactory, as judged from the recovery of total cholesterol. Individual rat serum values were available from the previous work, and the cholesterol content of the total lipid fractions of the present study averaged for the 9 groups 98.6 % of the values expected.

The chromatographic separation of the cholesterol esters from the other lipid components was carried out essentially according to FIL-

**Table I.**

*Rat serum lipid composition.*

| Type of oil | Daily dose mg | Chol. esters mg % | Free chol. mg % | Total. lip. mg % | Non. chol. lip. mg % |
|---|---|---|---|---|---|
| Control group ..... | 0 | 320 | 87 | 920 | 513 |
| Methyl linoleate .... | 20 | 334 | 81 | 880 | 460 |
| | 40 | 245 | 61 | 800 | 494 |
| | 80 | 242 | 33 | 720 | 445 |
| Methyl linolenate ... | 40 | 183 | 44 | 640 | 403 |
| | 100 | 180 | 21 | 535 | 334 |
| Cod liver oil ..... | 20 | 214 | 43 | 680 | 423 |
| | 40 | 167 | 24 | 560 | 369 |
| | 80 | 171 | 20 | 625 | 434 |
| Stock diet ..... | | 74 | 24 | 420 | 323 |

LERUP and MEAD (1953). The silica gel was obtained from The British Drug Houses Ltd. Prolonged elution with 1 % ethyl ether in pet. ether would in addition to the cholesterol esters also bring triglycerides in solution, as noted from the appearance of radioactivity in the later fractions when some $C^{14}$-labelled tristearin (Amersham) had been added to the lipid mixture. The cholesterol esters appeared from about the 2. to the 7. column volume, and these fractions were accordingly pooled. No more than 2 % of the triglycerides were found in these same fractions. The volume was again reduced to a few ml, and the ester fractions stored under $N_2$ in a weighed glass tube until alkali isomerization. Weighing of the solvent-free lipid fraction was carried out immediately before isomerization. For a more reliable determination of the amount of cholesterol ester present, 0.5 ml of n-hexane was added, and two 5 $\mu$l aliqots taken for cholesterol determination. The ester weights were then found by multiplication with the factor 1.69, the factor appropriate for cholesterol stearate. (The corresponding factor used for finding the acid weights was 0.745.) The ester weights computed were, in most instances, sufficiently lower than those directly obtained to indicate the presence of small amounts of some extra material of unknown nature in the pooled fractions.

Alkali isomerization was carried out on a micro-scale, according to HERB and RIEMENSCHNEIDER (1953). 1.5—2.5 mg of ester contained in 100 $\mu$l aliqots of the above solution was placed in the small glass cups, the solvent evaporated, and the cup introduced into the isomerization mixture. k-values at 233, 268, 315, 346, and 374 m$\mu$ were computed, using the fatty acid weights obtained from the cholesterol determinations. The computations thus immediately gave the polyenoic acid contents as % of total ester acids. The formulae used were based on those of HAMMOND and LUNDBERG (1953), assuming equal amounts of

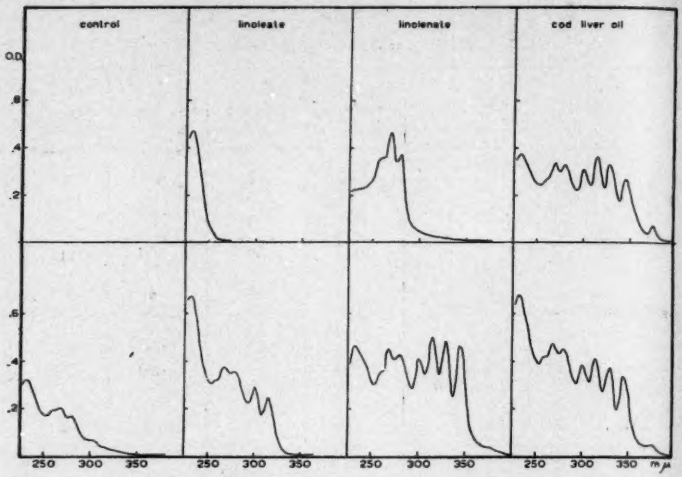


**Fig. 1. Alkali isomerization spectra, reproduced from Beckman DK recordings. The upper half gives the spectra for 5 mg/l of linoleic and linolenic acid, and for 25 mg/l of cod liver oil. The bottom half gives the spectra for the corresponding cholesterol esters, at 100 mg ester per liter, taken from the top dosage level groups.**

$C_{20}$- and $C_{22}$-pentaenoic acid. Some of the dienoic acid values of Table II, and to a lesser extent the trienoic values, may be somewhat too large, due to an extraneous absorption in the lower region of the ultraviolet that could not be properly corrected for.

## Results and Discussion.

The hydrogenated coconut oil-cholesterol regimen resulted in the serum lipid picture given in Table I (control group). As compared to data for our stock animals, it is seen that both free and esterified cholesterol are considerably elevated, and the relative contribution of ester cholesterol somewhat increased rather than decreased. One also notes a definite rise in the non-cholesterol lipid level. This rise is however not sufficient to prevent the cholesterol esters from making up a higher proportion of the total lipid than in the stock animals. 14 days of supplementation of the diet with 20—100 mg of the poly-unsaturated oils denoted resulted in a marked drop in both free and esterified cholesterol, the effect increasing with the dosage and varying with the nature of the

**Table II.**

*Polyenoic fatty acid composition of rat serum cholesterol esters.*

| Type of oil | Daily dose mg | % of chol. ester fatty acids as | | | | | Total poly-unsaturation[1] | % cholesterol depression[2] |
|---|---|---|---|---|---|---|---|---|
| | | dien | trien | tetraen | pentaen | hexaen | | |
| Control group ..... | | 3.7 | 3.2 | 0.9 | 0.5 | 0.7 | 26 | 6 |
| Methyl linoleate .... | 20 | 6.0 | 4.0 | 2.0 | 0.5 | 0.6 | 38 | —1 |
| | 40 | 7.3 | 5.0 | 4.7 | 1.2 | 1.4 | 63 | 22 |
| | 80 | 10.0 | 3.9 | 8.2 | 0.9 | 0.8 | 74 | 41 |
| Methyl linolenate .. | 40 | 3.8 | 4.1 | 2.4 | 7.1 | 1.7 | 75 | 52 |
| | 100 | 2.7 | 1.8 | 2.2 | 13.1 | 3.1 | 104 | 57 |
| Cod liver oil ..... | 20 | 4.6 | 5.5 | 2.0 | 1.6 | 1.4 | 50 | 39 |
| | 40 | 9.2 | 5.7 | 2.2 | 3.4 | 2.0 | 73 | 55 |
| | 80 | 7.0 | 3.4 | 3.0 | 8.2 | 3.6 | 99 | 58 |
| Stock diet ..... | | 18.2 | 1.9 | 45.6 | 3.7 | 1.0 | 250 | |

[1] % dien. 2+ % trien. 3 . . . ;

[2] reproduced from Haugr and Nicolaysen (1958 c).

supplement. Linolenic acid and cod liver oils are, as already reported, particularly potent depressors of the cholesterol lipid concentrations under the conditions of the experiment. Total lipids show a concomitant decrease, which is only partly accounted for by the decrease in cholesterol and cholesterol esters. This is also observed in man, *e. g.* AHRENS *et al.* (1957). Cholesterol esters as % of total lipids show no definite trend of change, and remains in average 32 %, while there is a small rise in the proportion of cholesterol that is esterified, a rise from 70 to about 85 %.

Fig. 1 and Table II contain information on the fatty acid composition of the cholesterol ester fractions. It is evident that the pronounced effect on cholesterol concentration is accompanied by an equally pronounced effect on the nature of the fatty acids linked to cholesterol. The degree of total poly-unsaturation is raised to a level of 3 to 4 times that of the control group. Whether the unsaturation will increase to the high level of the stock animals if the treatment is continued beyond 14 days is not known. It is likely that the load of saturated fat and cholesterol would tend to keep the unsaturation subnormal even at equilibrium. This load gives, in the fatty acid deficient control group, rise to a lower degree of poly-unsaturation than has been reported for animals on a fat and cholesterol-free diet. MUKHERJE *et al.* (1957) found 9 % dienoic acid in deficiency and a total poly-unsaturation of 53, compared to 23 % and 112 in a parallel group where the diet had been supplemented with 200 mg linoleate daily for 15 weeks. Considerably higher degrees of unsaturation were found by KLEIN (1957) under similar conditions.

Linoleate, linolenate, and cod liver oil each affect the cholesterol ester fraction in a characteristic fashion. Linoleate supplementation increases the diene content, as one would expect. But even more marked is the increase in tetraene. This is probably a manifestation of the well known transformation of linoleate to arachidonate (WIDMER and HOLMAN 1950, STEINBERG *et al.* 1956). In the present experiment the arachidonate formation is actually most clearly revealed in the stock animals, which received 5 % peanut oil in their diet, an oil containing linoleic acid as the only poly-unsaturated acid. No significant increase in trien, pentaen, or hexaen is noted with the linoleate treatment. With linolenate, on the other hand, it is the pentaenoic and hexaenoic acids that are affected. A very efficient conversion of linolenate to pentaenoic acids appears to have taken place, leaving in fact the trienoic

acid content largely unaffected. Evidence for such a transformation has also been reported by WIDMER and HOLMAN (1950). Pentaenoic formation appeared to be restricted to blood and kidneys, whereas hexaenoic acid increased in a number of rat organs upon feeding of linolenate. 3,6,9,12,15-$C_{20}$ and $C_{22}$-pentaenoic acids (numbered from the terminal methyl) have been found in beef liver phosphatides, and a $C_{22}$-hexaenoic acid with the probable double bond system 3,6,9,12,15,18 has also been isolated from the same source (KLENK 1956). Since linolenic acid is 3,6,9-$C_{18}$-trienoic acid, there is here a structural relationship of the same nature as between linoleate and arachidonate, the latter both having the 6,9-unsaturation with no double bond in the 3 position. The biosynthesis of the highly unsaturated acids of the linolenic acid type may then well follow the same pattern as is being established for arachidonate (MEAD and HOWTON 1957). 3,6,9,12-$C_{18}$-tetraenoic acid would be a likely intermediate in the formation of the pentaenoic and hexaenoic acids. Table II does in fact indicate the presence of some extra tetraenoic acid upon supplementation with linolenate.

The above mentioned tetraenoic, pentaenoic, and hexaenoic acids of the 3,6,9-type have by KLENK and co-workers been found to be the predominant type of poly-unsaturated acid in cod liver oil and other marine oils. They do, however, also contain significant amounts of dienoic and trienoic acids. The broad effect on all the polyenoic constituents of the cholesterol esters upon cod liver oil supplementation is therefore not unexpected. When evaluating the effect of cod liver oil it should be borne in mind that only 35 % of the acids contained are poly-unsaturated, these acids thus having a very high biopotency. Not unlike what is known for arachidonic acid, the organism here appears to prefer the ready made highly unsaturated acid. The transformations from the lower acids, although they can be carried out, appear to be rate limiting.

When the degree of unsaturation is correlated with the cholesterol depressive effect obtained in the same group, a final interesting feature appears. Comparing the 80 mg linoleate group with the 40 mg linolenate and cod liver oil groups, it is seen that about equal degrees of poly-unsaturation is accompanied by somewhat greater drops in cholesterol when this unsaturation is due more to acids of the 3,6,9-type than to those of the 6,9-type. In addition to this comes the fact that a certain dose of linolenic acid pro-

duced a larger degree of unsaturation than the same dose of linoleic acid. In the phase of cholesterol metabolism here studied and under the present conditions, acids of the 3,6,9-type thus seem to play a more active role than those of the 6,9-configuration.

The findings provoke the question whether the increased poly-unsaturation in the cholesterol esters is directly involved in the causation of the cholesterol depression, or whether it merely reflects a general change in the lipids, a change leading to increased activity of the catalytic system responsible for the breakdown of cholesterol. This question is presently the basis for further experimentation.

## Summary.

The regimen of 10 % hydrogenated coconut oil—1 % cholesterol resulted in high serum concentrations of both free cholesterol, cholesterol esters, and non-cholesterol lipids. Supplementation of this diet for 14 days with 20—100 mg daily of poly-unsaturated oils resulted in pronounced drops in these three variables. These drops were accompanied by characteristic changes in the polyenoic fatty acid composition of the cholesterol esters. Linoleate supplementation resulted in the appearance of more dienoic and tetraenoic acid in the esters, linolenate particularly in the appearance of pentaenoic, but also of some hexaenoic acid. Cod liver oil had an effect on all the polyenoic constituents, and the magnitude of this effect, referred to the actual content of polyenoic acids in the oil, was by far the strongest. This indicates a preference in the organism for the ready made poly-unsaturated acids. The largest reductions in total cholesterol were correlated with a preponderance of the 3,6,9-type unsaturation in the esters. Linolenic acid related polyenoic acids thus appear to play a more active role in cholesterol metabolism under the present conditions than linoleic and arachidonic acid.

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Acta physiol. scand. 1959. *45*. 190—210.

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# On the Role of Osmotic Water Transport in the Secretion of the Aqueous Humour.[1]

By

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Received 11 October 1958.

**Abstract.**

AURICCHIO, GIACINTO and ERNST H. BÁRÁNY. On the role of osmotic water transport in the secretion of the aqueous humour. *Acta physiol. scand.* 1959. *45*. 190—210. — Part of the water of a hypertonic secretion will enter passively along the osmotic gradient between blood and secretion. The aqueous humour is hypertonic in several species and it is often thought that all of its water enters as a consequence of the hypertonicity. To investigate this point, the osmotic pressure difference aqueous-plasma and the mobility of water under osmotic forces across the barrier between aqueous and plasma have been measured in anaesthetized cats.

The thermo-electric vapour pressure method was used. In steady state, the aqueous of the posterior chamber in cats was about 5 mOsm/l hypertonic to plasma. With the secretion rate of 15 $\mu$l/min water mobility would have to be about 3 $\mu$l/min/mOsm/l in order to explain the rate of water secretion by osmotic attraction.

In order to measure the actual water mobility, sudden changes in the osmotic pressure of the plasma were induced

[1] Presented in part at the 3rd Glaucoma Conference of the JOSIAH MACY, Jr. Foundation at Princeton N. J., Jan. 8—10, 1958.

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by anisotonic injections and the initial rate of volume change of the intraocular contents correlated with the (measured) degree of anisotonicity of the plasma. The mobility found was far below 3 $\mu$l/min/mOsm/l. Hence, water movement is not due to osmotic attraction by the bulk of the aqueous.

It is stressed that osmotic pressure measurements in vitro can be quite misleading when applied to living systems. In vitro methods use perfect membranes or their equivalents, which have no certain counterpart in vivo. Since in the living organism a steady state concentration difference can be upheld even across a leaky membrane, the magnitude and even direction of the osmotic force existing in vivo can differ markedly from that measured in vitro.

The nature of the forces moving the water of glandular secretions is still poorly understood. One view is, that the secretory forces act on certain solutes and that water is carried along by osmosis, *i. e.* that it moves because of an activity gradient caused by the transport of solutes. The secretion of the aqueous humour into the posterior chamber is a case in point. Ever since the discovery of an appreciable osmotic gradient between aqueous and plasma by BENHAM, DUKE-ELDER and HODGSON in 1938, it has been natural to assume that the maintained hypertonicity of the aqueous is an important cause for the continuous flow of the aqueous humour and hence for the maintenance of intraocular pressure. The hypertonicity of the aqueous humour is of the order of 5 mOsm/l, which corresponds to about 100 mm Hg at body temperature. Together with the capillary filtration pressure this represents a potential driving force which would seem sufficient to explain not only the normal intraocular pressure of about 20 mm Hg but also the high pressures observed in glaucoma. This is the view taken by many recent authors (see for instance DUKE-ELDER 1949 and KINSEY 1950).

Recent experiments with acetazolamide in this laboratory (AURICCHIO and WISTRAND 1958) have demonstrated that this drug, which causes considerable changes in net intraocular fluid production, has this effect without reducing the osmotic difference between aqueous and plasma. This finding led us to doubt that osmotic forces are dominant in transferring fluid into the eye. We have tried, therefore, to reinvestigate their importance.

The present paper consists of two sections. In the first, the

steady state osmotic pressure difference between aqueous humour and plasma is re-examined. It is confirmed that the anterior chamber aqueous in rabbits and the posterior and anterior chamber aqueous in cats are hypertonic with respect to the plasma. In the second section, an attempt has been made to measure the mobility of water across the blood-aqueous barrier under the action of osmotic forces. It is found that the mobility of water is too low to account for the formation of the aqueous under the osmotic gradient physiologically present between the bulk of this fluid and the plasma.

## I.

### *The steady state osmotic pressure difference between aqueous and plasma.*

#### 1. *Rabbit Experiments.*

Direct measurements of this difference with the Hill-Baldes thermoelectric vapour pressure method have previously been made by KINSEY (1951), who found the aqueous hypertonic to plasma by 3 mM/l equivalent of NaCl and in a small number of animals by ROEPKE and HETHERINGTON (1940), who obtained about half this value. Current work in this laboratory has amply confirmed Kinsey's results (AURICCHIO 1958, AURICCHIO and WISTRAND 1958). As stressed by DAVSON (1956) a possible source of error in all such measurements is the difference in protein concentration between the two fluids to be compared. KINSEY has tested this point by adding electrodialyzed crystalline bovine serum albumine to NaCl-solution and measuring its influence on the osmotic pressure. No abnormal effect was found. As a further control, we have made the following experiments in which the aqueous is compared with an in-vivo-dialysate of peritoneal fluid.

Small bags of 1/4 inch Visking Nojax cellophane tubing supported from within by a polyethylene spiral, were filled with $H_2O$, 0.9 % or 1.8 % NaCl and implanted into the peritoneal cavity of rabbits. Strict aseptic precautions were used. Moreover, the animals received 400,000 units of procaine-penicillin intramuscularly after the operation. Three to four days later, the animals were anaesthetized with allyl-isopropylbarbituric acid (Numal® Roche) i.v. and given 30 mg heparin in 0.9 % NaCl i.v. Thirty minutes later aqueous humour samples were taken, arterial

**Table I.**

*Peritoneal dialysate, anterior aqueous humour and plasma compared in the rabbit. Each value is the mean of two determinations with different thermopiles.*

| Date | Duration of dialysis | Initial contents | | Difference, mOsm/l | | | Protein mg/100 ml |
|---|---|---|---|---|---|---|---|
| | Days | ml | % NaCl | Aqueous-Dialysate | Aqueous-Plasma | Plasma-Dialysate | |
| 4/11 | 3 | 1 | 0.9 | + 6.1 | + 5.1 | + 2.0 | not tested |
| 5/11 | 4 | 1 | 0.9 | + 8.5 | + 6.5 | + 3.8 | » » |
| | | 0.3 | 0 | + 2.9 | + 7.6 | + 2.5 | 300 |
| 8/11 | 3 | 0.3 | 1.8 | + 6.5 | | — 1.5 | 30 |
| | | 0.3 | 0 | + 4.2 | | — 0.9 | 0 |
| 9/11 | 4 | 0.3 | 1.8 | + 5.2 | | | 0 |
| 20/11 | 3 | 0.3 | 0.9 | + 2.4 | + 2.9 | | 0 |
| 21/11 | 4 | 0.3 | 0.9 | + 6.1 | + 5.2 | | 30 |
| | | | | + 5.2 | + 5.5 | | |

heart blood removed anaerobically and the cellophane bags recovered. There was never any increased fluid in the peritoneal cavity, but sometimes a thin coating of fibrin on the outside of the cellophane bags. The blood was immediately centrifuged under liquid paraffin and the osmotic pressure differences between plasma, dialysate and aqueous measured using two thermopiles for each reading and taking the mean of the results. The technique in current use in this laboratory has been described (AURICCHIO 1958, AURICCHIO and WISTRAND 1958). The dialysates were tested semiquantitatively for protein using Albustix® reagent strips (Ames Co., Elkhart, Ind., USA). Table I shows the results.

They support the contention of KINSEY that the rabbit anterior chamber aqueous is hypertonic to the plasma by about 5 mOsm/l and support the validity of results obtained by direct comparison of plasma and aqueous with the thermoelectric method.

2. *Cat Experiments.*

There is only a single measurement reported for this animal. BENHAM et al. (1938) found the anterior chamber aqueous hyper-

**Table II.**

*Osmotic pressure difference aqueous-plasma in the cat. Each value is the median of 3 determinations with different thermopiles.*

| Cat | Anaesthesia | Heparin | Nature of Blood | Excess osmotic pressure in aqueous, mOsm/l | |
|---|---|---|---|---|---|
| | | | A = arterial V = venous | Anterior chamber | Posterior chamber |
| 21 | Ether-chloralose | in vitro | A | 4.9 | — |
| 25 | » | » | A | 1.3 | — |
| 26 | » | » | A | 0.5 | — |
| 30 | » | » | A | 2.6 | — |
| 29 | Numal | in vivo | V | 2.0 | — |
| 5 | » | » | A | 2.1 | — |
| 8 | » | » | V | 1.6 | 1.6 |
| 6 | » | » | A | — | 6.1 |
| 7 | » | » | V | — | 7.2 |
| 9 | Ether-chloralose | » | V | — | 4.9 |
| 10 | » | » | V | — | 4.9 |

tonic by 4.5 mM/l equivalent NaCl to serum, in a cat under pentobarbital anaesthesia. We have made a number of such measurements. Some cats were anaesthetized with chloralose, 80 mg/kg i.p., after induction with ether. The induction was produced without excitement. Moreover, in order to allow the normal osmotic relationships to be reestablished, the samples were taken 60—90 min after induction of anaesthesia. Aqueous was taken from the anterior chamber. Blood was taken from the femoral artery with a syringe moistened with 3 % heparin in 0.9 % NaCl. The dead space of syringe and needle was 0.09 ml. The blood sample was 3 ml, which dilutes the heparin solution some 30 times. The osmotic pressure or the heparin solution proved to be 30 mOsm/l hypertonic to plasma. Thus, the osmotic pressure of the plasma could have been increased 1 mOsm/l through the heparin in these experiments. The plasma was separated from the cells immediately after withdrawal by centrifugation under liquid paraffin and osmotic pressure measurements begun within one hour.

Since the values obtained in these experiments possibly are about 1 mOsm/l too low because of the heparin solution used,

a number of experiments were made with the cats heparinized in vivo. The animals were anaesthetized with 50 mg/kg of Numal® i.p. without struggling and left alone one hour on a heated table. They were then heparinized with 10 mg heparin in 0.9 % NaCl and 30 min later arterial or venous blood and anterior aqueous humour samples were taken. The results of both these groups of experiments are shown in Table II.

Posterior chamber punctures in the cat have not been reported before. We have made a small number in which the posterior chamber was entered with a fine needle about 6 mm behind the limbus. The results show that the aqueous humour of the posterior chamber is also hypertonic with respect to plasma.

It would seem that the hypertonicity of the cat's aqueous is in the same range as that of the rabbit.

### 3. *Discussion of section I.*

What then is the significance of the hypertonicity of the aqueous under normal conditions?

An osmotic pressure difference is a measure of the tendency of water to flow. But the pressure difference between two solutions is dependent on the relative "leakiness" of the membrane to the solutes present on both sides, a perfect semi-permeable membrane being one which is permeable to the solvent only. Moreover, the rate and direction of water flow is also dependent on electrical gradients if such are present. It is not certain, therefore, that the hypertonicity found in vitro with a perfect membrane (or its equivalent, the air space, as employed in the vapor pressure technique), and in the absence of electrical gradients indicates correctly the tendency for water to flow in vivo. The blood-aqueous barrier may exhibit properties which are far from those of perfect semi-permeable membranes. Moreover, an appreciable electrical potential gradient may exist between blood and aqueous. It is, therefore, possible that the hypertonicity of the aqueous with respect to plasma as measured by the vapour pressure method does not correctly indicate the tendency of water to move into the eye in vivo.

If the aqueous and plasma differed only in water content, *i. e.* all of the solutes were present in relatively the same concentration, the osmotic pressure difference as measured by the vapour pressure method would represent the maximum possible.

However, if the relative concentration of the solutes in the two fluids to be compared differed sufficiently widely then the osmotic pressure difference as measured by the vapour pressure method might even be an underestimate of the in vivo tendency for water movement due to selective permeability effects. It is improbable, however, that this is so in the present case, since the plasma and the aqueous are actually closely similar in their composition, especially with respect to the cations. But the osmotic pressure difference determined in vitro can very well be larger than that effective in vivo. In the following, the in vitro osmotic pressure measurements will be considered as if they gave a correct picture of the situation in vivo. Nonetheless, the above reservation should be kept in mind.

## II.

### *An upper limit for the mobility of water between blood and aqueous.*

1. *Principle of the method:* The anterior chamber of an anaesthetized cat is connected to a horizontal burette. The height of the burette over the eye is adjusted so that fluid neither leaves nor enters the eye under steady state conditions. When the osmotic pressure of the blood is changed by the injection of a suitable anisotonic solution, water is shifted across the barrier between the blood and the intraocular tissues and fluids and the contents of the eye bulb change in volume. Since the pressure in the eye is kept nearly constant by the horizontal burette, the capacity of the globe and the outflow at the chamber angle do not change appreciably, and the amount of fluid entering or leaving the burette is a direct measure of the volume change of the contents of the globe. If all the volume change is attributed to the aqueous humour and this change divided by the mean anisotonicity caused by the injection, an upper limit is obtained for the mobility of water across the barriers between blood and aqueous.

2. *Experimental:* Cats weighing 2.5—3.5 kg were anaesthetized with 50 mg/kg Numal® i. p. occasionally supplemented with 5—10 mg/kg pentobarbital (Nembutal®). They were tracheotomized, one femoral vein cannulated for injections and the right brachial artery or one external jugular vein connected with a strain-gage manometer record-

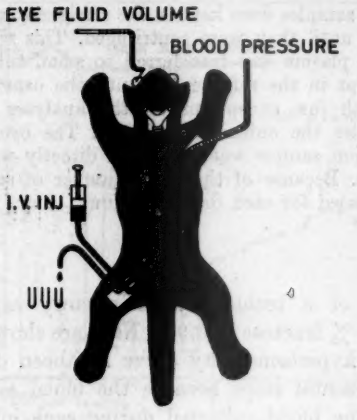



Fig. 1. Diagram of set-up for isotonic recording of intra-ocular volume changes.

ing on an ink-writing oscillograph. A lateral canthotomy was performed and the lids were held apart by sutures. After heparinization, 10—20 mg/kg, a polyethylene tube (length 180 mm, bore 0.6 mm) was pushed up a few centimenters into the abdominal aorta through the femoral artery and closed with a short length of narrow rubber tubing and a clip. The cornea was anaesthetized with 2 % lidocaine without adrenaline and the anterior chamber cannulated with a sharp needle, bore 0.5 mm, leading to a horizontal burette by means of a narrow polyvinylchloride tubing. The horizontal burette, internal diameter 0.96 mm, contained physiological saline and was calibrated in $mm^3$. Its capillary attraction was 3 cm $H_2O$ and the frictional resistance of the whole arrangement 0.5 cm $H_2O$ per $mm^3$/sec. The height of the burette was adjusted so that the drift of the meniscus was minimal or, in some experiments, slowly towards the eye, then the collection of blood from the femoral artery was begun and after $^1/_2$—1 min. of collection, a slow intravenous injection of 12.5 % fructose or 25 % saccharose in 0.9 % NaCl was started. In hypotony experiments, 0.3 % or 0.5 % NaCl was injected instead.

All injections were given by hand; the amount was 2 ml/kg body weight of the sugar solutions and 2 or 4 ml/kg of the hypotonic solutions. The rate of injection was approximately 4 ml/min. Starting about 1 min before the injection, blood was continuously collected in centrifuge tubes which were shifted every 12—30 sec. The rate of blood flow was 2—3 ml/min. Starting several min. before the injection a separate observer continuously read the position of the meniscus in the burette to the nearest 0.5 $mm^3$ into a tape recorder from which the readings were later timed with a stop watch and recorded graphi-

cally. The blood samples were kept in the refrigerator in tubes covered with Parafilm® until they were centrifuged. This was done as soon as possible. The plasma was transferred to small tubes covered with parafilm and kept in the refrigerator until the osmotic pressure was determined. With few exceptions all the analyses were completed within 24 h. after the animal experiment. The osmotic pressure of each post-injection sample was compared directly with the last pre-injection sample. Because of the large number of samples, only one thermopile was used for each determination.

3. *Results.*

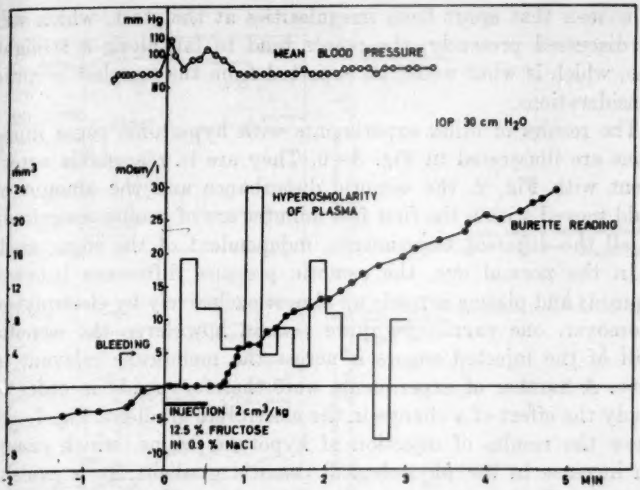
The results of a technically satisfactory experiment using 2 ml/kg of 12.5 % fructose in 0.9 % NaCl are shown in figure 2 A.

The plasma hyperosmolarity curve has been drawn as a succession of horizontal steps because the blood samples analysed contained all the blood collected during each interval and thus yield a mean value for the osmolarity. The time delay in the catheter is only 1—2 sec at most and has been neglected. The strong fluctuations shown by the curve are mainly due to recirculation and incomplete mixing during the first few minutes after the intravenous injection, though the large negative value recorded for the period 2.6—2.8 min certainly must be erroneous. The mean hyperosmolarity during the first 3 min is 9 mOsm/l and is obtained by dividing the surface under the curve with the time.

The volume displacement curve, marked 'Burette reading', shows that 2—3 $mm^3$ leave the burette even prior to the injection period, at the start of the withdrawal of blood. Following this, there is no fluid movement during the first 40 sec after the start of the hypertonic injection, in spite of the well-developed hypertonicity of the plasma. After this period of delay, fluid starts to leave the burette for the eye and the curve rises. At the end of the first 3 min counted from the start of the injection, 16 $mm^3$ have left the burette.

The blood pressure curve shows initial fluctuations, probably caused by the injection, but a return to the initial value before the end of the recording.

The relation between fluid shift and osmotic disturbance of the plasma is more clearly brought out if the volume displacement is plotted not against time but against the time integral of the osmotic disturbance, the accumulated osmotic push or pull. Fig. 2 B shows this relation for the experiment of Fig. 2 A.




Fl. leaving eye

Fig. 2 A. Changes in intra-occular volume (∘–∘–∘–∘), osmotic pressure of plasma (staircase curve) and mean arterial blood pressure (o-o-o-o) caused by the intravenous injection of 2 ml/kg body weight of 12.5 % fructose in 0.9 % NaCl.

Time is counted from the start of the injection. The mean blood pressure is the arithmetic mean of systolic and diastolic measured in the brachial artery.

Osmolarity values are in comparison with the last prae-injection sample. Positive values indicate hyperosmolarity of the post-injection samples.

The zero for the burette reading curve has been set at the position of the meniscus at the start of the injection. The rising values indicate that fluid leaves the burette for the eye.

IOP = intra-ocular pressure.

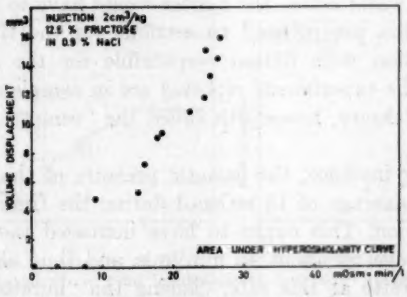



Fig. 2 B. Fluid displacement versus cumulated osmotic pull. Data of Fig. 2 A replotted.

It is seen that apart from irregularities at the start, which will be discussed presently, the points tend to fall along a straight line, which is what would be expected from the simplest osmotic considerations.

The results of other experiments with hypertonic sugar injections are illustrated in Fig. 3—6. They are in reasonable agreement with Fig. 2, the osmotic disturbance and the amount of fluid moved within the first few minutes are of similar magnitude in all the different experiments, independent of the sugar used.

In the normal eye, the osmotic pressure difference between aqueous and plasma is made up almost exclusively by electrolytes. Moreover, one cannot be quite certain how large the osmotic pull of the injected sugars is across the membrane relevant in vivo. A number of experiments were therefore made in order to study the effect of a change in the electrolyte gradient. Fig. 7—11 show the results of injection of hypotonic saline, which causes an increase in the physiological osmotic gradient. It is evident that the fluid movements caused by these injections are very small and it has therefore been neither possible nor necessary to construct cumulated curves of the type of Fig. 2 B for these experiments. The plasma samples were only slightly hemolytic in these experiments.

### 4. *Discussion of section II.*

The rate of production of aqueous humour in the cat is close to 15 mm³/min (LANGHAM 1951). The osmotic pressure difference aqueous-plasma according to the measurements reported in section I is probably not larger than about 5 mOsm/l. Hence, the mobility of water across the barrier would have to be at least about 3 mm³/min per mOsm/l to account for the flow, if the osmotic attraction were indeed responsible for the movement of the water. The experiments reported are in complete disagreement with this theory, henceforth called the "osmotic attraction theory".

In Fig. 7, for instance, the osmotic pressure of the plasma is reduced by an average of 15 mOsm/l during the first 2 minutes after the injection. This ought to have increased the influx of water into the eye by about 45 mm³/min and fluid should have entered the burette at this rate, causing the "burette reading" curve to make a large bend downwards. In fact, however, there

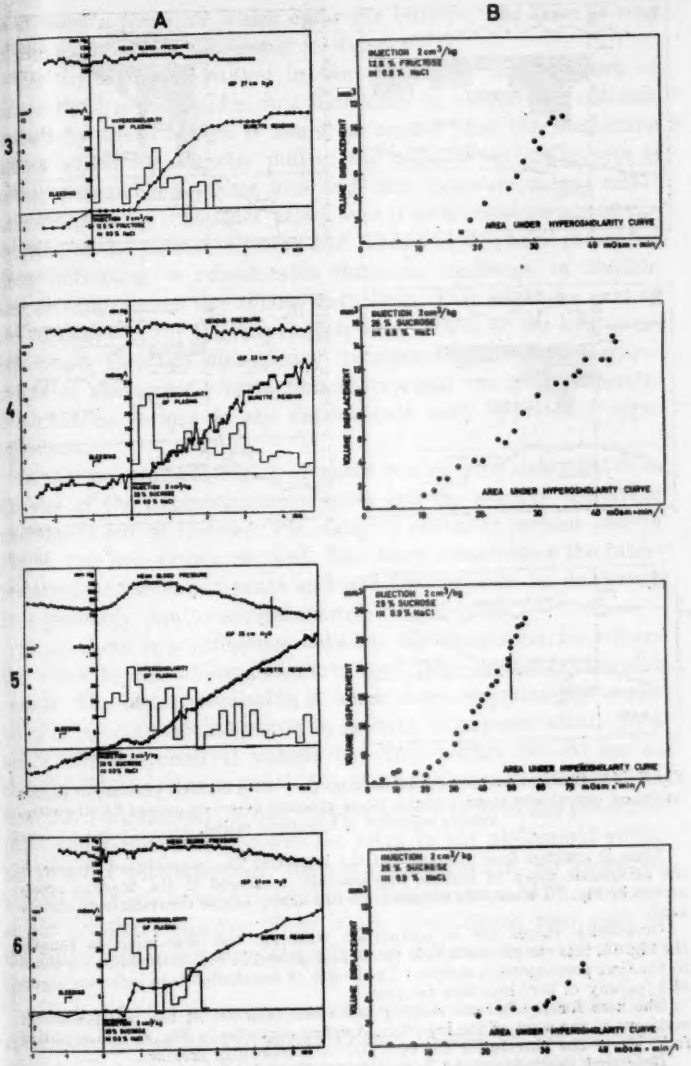



Fig. 3—6. See explanation of Fig. 2 A and B. In Fig. 4 the blood pressure was measured in the contralateral external jugular vein.

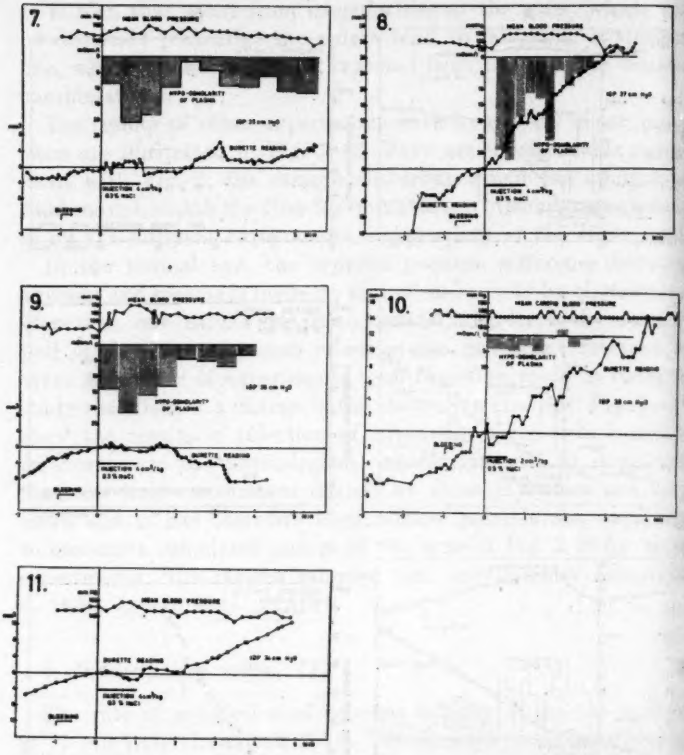



Fig. 7—11. Changes in intra-ocular volume (•–•–•–•), osmotic pressure of plasma (staircase curve) and mean arterial blood pressure (o-o-o-o) caused by the intravenous injection of hypotonic saline.

Time is counted from the start of the injection. The mean blood pressure is the arithmetic mean of systolic and diastolic measured in the brachial artery except in Fig. 10 where the common carotid artery of the contralateral side was used.

Osmolarity values are in comparison with the last prae-injection sample. (In Fig. 11, this sample was lost in the centrifuge and in Fig. 8, the same happened to the first post-injection sample.) The length of the shaded bars indicates degree of hypotony of post-injection samples.

The zero for the burette reading curve has been set at the position of the meniscus at the start of the injection. A rising curve (as in Fig. 8) indicates that fluid leaves the burette for the eye. IOP = intra-ocular pressure.

Note that the volume and hypotonicity of the injection in Fig. 10 was less than in the other experiments.

is at most a few mm³ which enter the burette. The same is true of the experiments illustrated in Fig. 8 to 11.

The hypotonicity caused in the plasma by the injection of dilute NaCl is mainly due to a deficiency in sodium and chloride caused by the dilution. It could be argued that the membrane across which the osmotic pull of the aqueous normally acts is rather permeable to these ions and that therefore only a small osmotic pressure difference arises over it as a consequence of the dilution of the plasma. KINSEY and REDDY (1958) have presented data indicating a considerable diffusion exchange of sodium and chloride across the ciliary epithelium. This might be part of the explanation for the surprisingly small effect of the hypotonic injections. But the discrepancy between the mobility requirements of the osmotic attraction theory and the present results is almost as serious in the experiments with hypertonic sugar injections.

In the experiments Fig. 2—5 there is a striking delay between the rise of the hyperosmolarity curve and the start of the water movement out of the eye. The delay is probably present also in Fig. 6, but less clearly marked. The delay complicates the interpretation of the experiments and will first have to be discussed. It is probably due to several contributing factors.

First, there is a difference between the circulation time from the veins to the abdominal aorta and that to the intraocular vessels. The latter circulation is much more sluggish. If fluorescein is injected intravenously in rabbits, it appears within 5—6 sec in the conjunctival vessels but only within 10—30 sec in those of the iris (BERGGREN 1956). In the cat the circulation time from the femoral vein to the carotid artery is 3—5 sec (SPECTOR 1956) and perhaps one or two sec more to the abdominal aorta, but in a cat with unusually light pupillary margin, we measured a delay of 16 sec between injection and appearance of fluorescence at the pupillary margin. Thus, there is no doubt that part of the initial delay is due to the effect of differences in circulation time.

A second factor probably contributing to the delay in the fluid movement is the hemodynamic change caused by the injection in some experiments. The systemic venous pressure was recorded in three experiments (Fig. 4 and two not shown) with injection of 2 ml/kg of either 12.5 % fructose or 25 % saccharose in 0.9 % NaCl. The rise has been at most 2 mm Hg, which is negligible

from the point of view of the eye since the episcleral and the intraorbital venous pressures are much higher than the systemic even after the rise. But the arterial pressure rise in Fig. 2 for instance, could be responsible for part of the delay. It might cause an increase in the filling of intraocular vessels and mask a simultaneous osmotically induced decrease in volume. On the other hand, in Fig. 3, where the delay is exceptionally well marked, blood pressure changes were minimal.

Besides a passive dilatation of the intraocular vessels, the hypertonicity *per se* could conceivably cause a vasodilatation in the eye. This would cause a delay. If hyperosmosis of the plasma moves water into the vessels but the vessels dilate so as to accommodate the fluid abstracted from the tissues of the eye, then hyperosmosis causes no visible fluid movement in the burette. It is possible that a factor of this kind plays a part in the delay.

A third and probably major factor contributing to the delay is the presence of a perivascular, extracellular pool of tissue fluid — *e. g.*, in the ciliary processes — which does not equilibrate instantaneously with the blood. This pool would be expected to lose some water to the blood initially because of the resistance offered to the diffusion of sugar by the capillary walls, but the main shrinkage will occur only after the pool has become hyperosmotic throughout. Thus, the presence of the extravascular pool will cause an initial part of the hyperosmolarity of the plasma to be lost from the point of view of fluid movement, but when the plasma hyperosmolarity disappears, the pool will cause a continued fluid movement instead. Since our experiments were finished before the plasma hyperosmolarity had disappeared, the osmotic attraction "lost" at the beginning is probably not wholly recouped at the end. Matters are probably similar with respect to intraocular vasodilatation caused by the hypertonicity.

A final reason for part of the delay could be irregular inertia and stickiness in the capillary tube used for recording the fluid movement. True inertia was negligible with the low rates of fluid movement encountered, but stickiness may have played a role, for instance, in the experiments of Fig. 4 and 6, where fluid movement is somewhat irregular. To minimize the effect of stickiness, we deliberately kept the burette pressure too high in the experiment described by Fig. 5 so that fluid was moving into the eye even before the injection. There was less delay in this case than in any other. However, the results are the same.

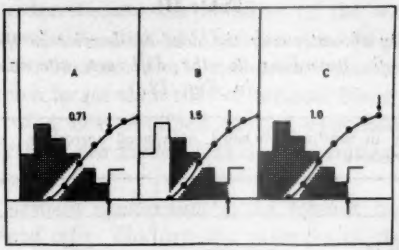



Fig. 12. To illustrate how Table III was calculated.

Staircase curve represents hyperosmolarity of plasma, •—•—•—• fluid displacement.
Shaded part of staircase curve indicates the part of the osmotic transient taken into regard when estimating osmotic mobility of water.

From the simplest osmotic considerations, the amount of water moved should be proportional to the area under the time-hyperosmolarity curve, but, as mentioned, the presence of the delay complicates quantitative interpretation.

However, it is possible to make certain limiting assumptions from which one can deduce an upper limit for the mobility of the water. This is illustrated by Fig. 12, which represents a model of our experiments. Starting from the staircase-shaped hyperosmolarity curve, a water movement curve has been calculated, by making the water movement during each step proportional to the area under the curve two steps earlier. This mimics the purely mechanical delay caused by differences in circulation time, but not the other factors discussed above. The figure shows three ways of dealing with the experimental results. Alternative A compares water moved with total surface under the hyperosmolarity curve up to the same moment. It underestimates mobility of water (by a factor of 0.71 in the model). Alternative B overestimates mobility (by a factor of 1.5 in the model) by neglecting all the forces preceding the actual start of the water movement. Alternative C corrects completely for the purely mechanical delay in the model but cannot be assumed to correct equally well for the other causes of delay. We have, therefore, treated our data according to each of the three alternatives and table III shows the results.

**Table III.**

*Osmotic mobility of water over the blood-eye-barrier in the cat calculated from Fig. 2—6, according to the different alternatives illustrated in Fig. 12.*

| Expt. Fig. | Mobility in mm³/min/mOsm/l, computed according to alternative | | | Injection |
|---|---|---|---|---|
| | A | B | C | |
| 2 | 0.59 | 0.80 | 0.67 | fructose |
| 3 | 0.31 | 0.58 | 0.34 | » |
| 4 | 0.33 | 0.40 | 0.38 | sucrose |
| [1]5 | < 0.42 | < 0.45 | < 0.46 | » |
| 6 | 0.18 | 0.28 | 0.22 | » |
| | | Av. 0.50 | | |

[1] The rising slope of the volume curve before the injection has not been corrected for. Hence all values are overestimates. Because of the blood pressure changes, only the part of the experiment to the left of the vertical line across the blood pressure curve has been used. This line indicates when blood pressure has recovered its initial value.

The results indicate that the normal osmotic pressure difference is quite insufficient to account for the production of the aqueous humour. The mobility would have to be at least about 3 mm³/min per mOsm/l osmotic pressure difference to explain the physiological flow according to the osmotic attraction theory, while our experiments indicate a mobility of at most about 1/6 of this value. (The hypotony experiments indicate an even smaller fraction). Moreover, our experiment does not distinguish between water removed from the aqueous and water removed from the rest of the contents of the eye bulb. Therefore, the values found represent an upper limit for several reasons and the discrepancy between our results and the idea that water enters the aqueous as a result of excessive osmotic pressure in the aqueous is even more serious than the figures suggest.

Before discarding the osmotic attraction theory, however, it is necessary to discuss sources of error in the present experiments. In this discussion it will be assumed that the osmotic attraction theory holds.

*Leakiness of the blood-aqueous barrier for the injected substances.* This could make us overestimate the osmotic pull of the hypertonic

plasma and underestimate the mobility of the water. But it is extremely improbable that the leakiness would be smaller for fructose than for sucrose. The distribution volume of fructose in the body is much larger than that of sucrose. Since the values for the mobility of water obtained in the experiments involving fructose if anything are higher, leakiness cannot be an important source of error.

*Failure of osmotic equilibrium to be reached in vivo between plasma and blood cells.* The osmotic pressure of the plasma was measured usually only after 15—30 min of contact between the blood cells and the anisotonic plasma and osmotic equilibrium must have been established before the plasma was separated from the cells. It is possible that in vivo the time of contact was too short for full equilibrium between cells and plasma to be reached. This source of error would cause us to underestimate the actual anisotonicity of the plasma in vivo and to overestimate the mobility of water.

*Concentration changes due to movement of water.* When water is moved by osmotic forces, the concentration gradient tends to decrease until a new equilibrium is established. To minimize the effect of decreasing osmotic gradient, the rate of water movement was determined within the first few minutes after altering the osmolarity of the plasma. Actually, the curves relating water movement to surface under the time-hyperosmolarity curve do not show a decreasing slope even after four minutes. This may be seen from Fig. 4 b and 5 b. Hence, rapidity of attainment of osmotic equilibrium is not the cause of the low mobility of water observed.

*Haemodynamic changes.* The rate of bleeding was 0.5—1 ml/min/kg body weight, which corresponds to less than 2 % of the blood volume. During the experimental period, at most 10 % of the blood volume could have been lost. On the other hand, the injection of 2 ml/kg of about 3 × isotonic crystalloid solution will have caused a transient expansion of the blood volume by about the same amount. This probably is the reason for the quite moderate changes in blood pressure observed in most of the hypertonicity experiments. It is obvious from a comparison of the results in the different experiments that the haemodynamic changes could only have been of secondary importance in affecting the results.

It can be mentioned here that in a considerable number of

earlier experiments in rabbits and cats, we injected stronger sugar solutions and used shorter injection times. These injections caused marked haemodynamic changes which complicated the interpretation even if the results were the same; a very low water mobility.

Summarizing, it can be claimed that the present experiments show that the osmotic attraction of the *bulk* of the aqueous humour is not the force moving the water across the ciliary epithelium. Does this prove that not only the solutes but also water is acted upon directly by a secretory mechanism, perhaps related to pinocytosis, which transports a virtually isotonic solution? This was the conclusion drawn by the authors when the present paper was read at the 3rd Glaucoma Conference of the Macy Foundation. However, at the conference, Dr. ELMER BALLINTINE pointed out that passive water transport still could not be ruled out. If transport of a high concentration of the strategic solutes occurs into the depths of folds of the cell membrane of the free end of the cell ($\beta$-cytomembranes of SJÖSTRAND 1956, shown to be present in ciliary epithelium by HOLMBERG 1957), then passive water attraction and progressive dilution could occur while the fluid moves towards the free cell surface. In fact, if the process is that suggested by Dr. BALLINTINE and the folds are deep enough the fluid could be in its final state of dilution before it appears at the free surface of the cell. The fact that this final state is about 5 mOsm/l hypertonic in vitro does on not exclude the possibility of a virtual equilibrium, as discussed p. 196—197. In the steady state, the rate of volume secretion at the free end of the cell would then be proportional to the rate of osmole secretion into the depths of the folds and inversely proportional to the osmolarity of the plasma. This influence of the plasma concentration is not contradicted by our results. Since the volume of fluid present in the folds at any time is quite small, the instantaneous effects of plasma anisotonicity on fluid volume in the eye can only be very limited.

It appears, that both of the mechanisms suggested are compatible with our results and with the fact that inhibition of volume secretion by acetazoleamide occurs without change in tonicity. The question of how the water is carried during secretion therefore still is an open question.

## Summary.

1. The osmotic pressure difference between the aqueous humour of the anterior chamber and the plasma in rabbits has been measured with the thermoelectric vapour-pressure method. The aqueous is hypertonic by about 5 mOsm/l. It is equally hypertonic to an in-vivo dialysate of peritoneal fluid obtained by implanting cellophane bags into the peritoneal cavity.

2. In the cat, the aqueous humour of the anterior and of the posterior chamber is also hypertonic to the plasma. The hypertonicity of the posterior aqueous is close to 5 mOsm/l, that of the anterior aqueous possibly somewhat less.

3. A method for recording changes in the volume of the intraocular contents of the cat at constant pressure has been developed. Sudden considerable changes in the osmolarity of the plasma caused only small changes in this volume. Quantitatively, the mobility of water across the blood-aqueous barrier was altogether too small to explain the normal flow of aqueous as a consequence of the existing osmotic pressure difference between aqueous and blood.

4. It is stressed that osmotic pressure measurements in vitro may give quite misleading information as to the direction and magnitude of the osmotic force existing in vivo. The in vitro methods use perfectly semipermeable membranes or their equivalents while such perfection probably never is realised in vivo.

This investigation was supported by the Swedish Medical Research Council. We wish to thank Drs E. J. Ballintine, V. E. Kinsey and J. R. Pappenheimer for valuable discussions and Miss I. Dalhagen for technical assistance.

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Acta physiol. scand. 1959. *45*. 211—215.

Aus dem Physiologischen Institut d. Univ. Marburg/Lahn, Deutschland.

# Über die Übermittlung von Kälteschmerz.

Von

KURT BOMAN.

Eingegangen am 11. Oktober 1958.

**Abstract.**

BOMAN, K. On the mediation of cold pain. *Acta physiol. scand.* 1959. *45*. 211—215. — In the rat infraorbital nerve activity can be demonstrated in small nerve fibres by cutaneous temperatures below 25° C. The number of active nerve fibres is determined by the absolute cutaneous temperature. It is believed that these fibres mediate sensations of cold pain.

Die z. B. VON ALRUTZ (1897) und SCHRIEVER (1928 a, b) vertretene Auffassung, dass die beiden durch tiefe Hauttemperaturen hervorgerufenen Kälteschmerzqualitäten, heller und dumpfer Schmerz, durch ihre spezifischen Empfänger übermittelt werden, ist von mehreren Forschern angezweifelt worden. Nach den von LELE, WEDDELL und WILLIAMS (1954) und von SINCLAIR (1955) durchgeführten Untersuchungen sind die Empfindungsqualitäten des durch schmerzhafte Reize (Brennen, Frieren, Stechen, ätzende Säuren usw.) hervorgerufenen Schmerzes nicht voneinander scharf abgrenzbar. Die histologischen Untersuchungen über die Hautnerven (WEDDELL und SINCLAIR 1953 und WEDDELL, PALLIE und PALMER 1954) sprechen auch dafür, dass die von v. FREY (1895) aufgestellte Theorie über die Spezifität der Hautsensibilität nicht mehr aufrecht erhalten werden kann, weil auch

die freien Nervenendaufzweigungen Druck, Temperatur und Schmerz übermitteln können. Aus den vorliegenden Untersuchungen ergab sich, dass in der äusseren Haut tatsächlich neben den Thermoreceptoren kleinkalibrige Fasern vorkommen, in denen eine Impulsentladung bei tiefen Hauttemperaturen festgestellt werden kann.

Die Versuche wurden an 10 Ratten durchgeführt. Nach Einbinden einer Trachealkanüle wurde ein Auge enukleirt, der Nervenstamm des N. infraorbitalis in der Orbita wurde freigelegt und aufgesplittert. Die Aufzeichnung der Impulse und der Hautoberflächentemperatur erfolgte in üblicher Weise (HENSEL 1952). Die Abkühlung der Haut wurde durch abgekühlte Luftströme erzeugt, die auf die Haut aufgeblasen wurden.

Die Abb. 1. zeigt die stationäre Entladung mehrerer Temperaturfasern im N. infraorbitalis bei verschiedenen Hautoberflächentemperaturen (vgl. BOMAN 1958 a, b). Die Schwellenwerte der stationären Entladung der Temperaturfasern sind 15 und 38°. In Temperaturbereichen unterhalb von 25° Hautoberflächentemperatur ist das Vorkommen von kleinen, hochfrequenten Impulsen von einer Amplitude von 10—20 $\mu$V ersichtlich. Die Frequenz einer einzelnen Faser dieser Art ist in allen Temperaturbereichen unterhalb des jeweiligen Schwellenwertes immer dieselbe. Das in Tätigkeittreten von neuen Fasern dieser Art wird noch in Temperaturbereichen beobachtet, wo keine Dauertätigkeit der eigentlichen Thermoreceptoren mehr vorhanden ist.

Die Abb. 2 zeigt das Verhalten von Fasern dieser Art bei Erwärmung der Haut. Es wird beobachtet, dass die Entladung dieser Fasern bei 25° Hautoberflächentemperatur aufhört. Durch Erwärmen der Haut wird die Dauertätigkeit der eigentlichen Thermoreceptoren (grössere Impulse) gehemmt.

Wenn wir annehmen, dass die Intensität der Temperaturempfindung durch die pro Zeiteinheit an das Zentralorgan gelangende Anzahl der Thermoimpulse bestimmt wird, kann Folgendes angenommen werden: Die Intensität der Temperaturempfindung wird bis zum Maximum der stationären Entladung der Thermoreceptoren bei konstanten Temperaturen in den ganzen Nerven durch das in Tätigkeittreten von neuen Thermoreceptoren und durch die Zunahme der Entladung der einzelnen Thermoreceptoren bestimmt. In dem positiven Bereich der stationären Entladung im Gesamtnerven wird die Emp-

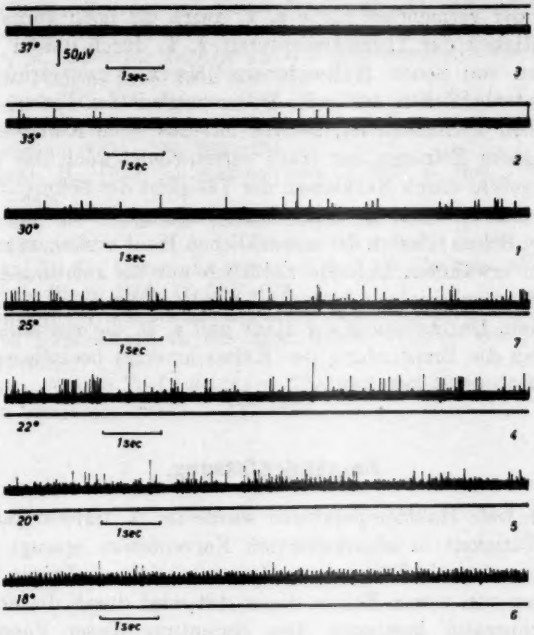


Abb. 1. Impulse von einer dünnen Präparation des N. infraorbitalis der Ratte bei verschiedenen konstanten Temperaturen. Das Vorkommen von Kälteschmerzimpulsen in Temperaturbereichen unterhalb von 25° ist ersichtlich. Die Zahlen bezeichnen die Reihenfolge von Registrierungen.


Abb. 2. Impulse von Kälteschmerzfasern und von Temperaturfasern bei Erwärmung der Hautoberfläche von 18 auf 25°. Das Aufhören der Entladung der Kälteschmerzfasern ist durch einen Pfeil markiert.

findung der vermehrten Kälte z. T. durch die noch vorhandene Dauertätigkeit der Thermoreceptoren, z. T. durch das in Tätigkeittreten von neuen Kälteschmerz(?)fasern hervorgerufen. In Temperaturbereichen, wo keine Dauertätigkeit der Thermoreceptoren mehr vorhanden ist, besteht nur das reine Kälteschmerzgefühl. Beim Erfrieren der Haut verschwindet auch das Kälteschmerzgefühl durch Nachlassen der Tätigkeit der Schmerzfasern.

Weil uns bisher noch keine Untersuchungen über die Thermo- bzw. Schmerzfasern der menschlichen Haut vorliegen, müssen die oben erwähnten Befunde natürlich nur als richtungsgebend für die Sinnesphysiologie betrachtet werden. In wieweit die Akklimatisation, Durchblutung der Haut und z. B. die zentralnervöse Steuerung die Empfindung des Kälteschmerzes beeinflussen, ist bisher noch nicht bekannt.

## Zusammenfassung.

Durch tiefe Hauttemperaturen wurde im N. infraorbitalis der Ratte Tätigkeit in kleinkalibrigen Nervenfasern erzeugt. Diese Fasern werden als Kälteschmerzfasern bezeichnet. Das in Tätigkeittreten von neuen Fasern dieser Art wird durch die absolute Hauttemperatur bestimmt. Die Bedeutung dieser Fasern als Übermittler des Kälteschmerzgefühls wird diskutiert.

## Summary.

Activity can be elicited in small nerve fibres of the infraorbital nerve in the rat by cutaneous temperatures below 25° C. The number of active fibres is determined by the absolute skin temperature. These fibres are believed to mediate sensations of cutaneous cold pain.

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Acta physiol. scand. 1959. 45. 216—219.

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# Die Einwirkung von Druckreizen auf spontantätige mechanosensible Receptoren der Haut.

Von

KURT BOMAN.

Eingegangen am 11. Oktober 1958.

**Abstract.**

BOMAN, K. The influence of pressure on the activity of spontaneous active cutaneous mechanosensible receptors. *Acta physiol. scand.* 1959. *45.* 216—219. — In the infraorbital nerve of the cat and the rat nerve fibres from cutaneous receptors with high specificity of mechanical stimuli can be demonstrated. These receptors are not influenced by thermal stimuli.

Die neuzeitliche elektrophysiologische Forschung über die Receptoren der äusseren Haut (MARUHASHI, MIZUGUCHI und TASAKI 1952, WITT 1958, BOMAN 1958) hat gezeigt, dass die von v. FREY (1895) vertretene Auffassung über die Spezifität der Hautreceptoren nicht mehr für richtig gehalten werden kann, — nach diesen Untersuchungen muss die Spezifität eines Hautreceptors als durchaus relativ betrachtet werden. Die neue histologische Forschung über die Hautnerven spricht auch mehr für einen indifferenten Aufbau der cutanen Receptoren als für eine Spezifität derselben (WEDDELL, PALLIE und PALMER 1954). In gewissen Hautreceptoren kann doch oft eine verhältnismässig grosse Spezifität elektrophysiologisch festgestellt werden. In den

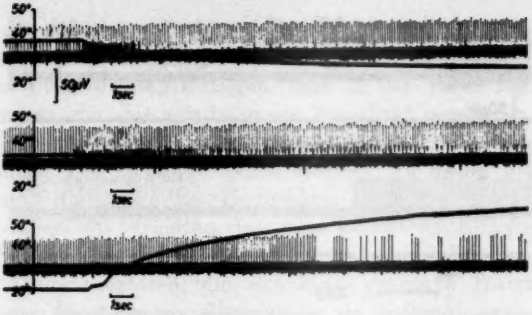


Abb. 1. Aktionspotentiale von einer einzelnen spontantätigen mechanosensiblen Faser und von einer einzelnen Temperaturfaser im N. infraorbitalis der Katze bei verschieden schnellen Kältesprüngen und bei einem Wärmesprung.

vorliegenden Versuchen wurde die Abhängigkeit der Tätigkeit der spontantätigen mechanosensiblen Hautreceptoren von der Intensität der Druck- und Temperaturreize untersucht, wobei die relativ grosse Spezifität dieser Receptoren festgestellt werden konnte.

Als Versuchstiere wurden Katzen und Ratten verwandt. Nach Einbinden einer Trachealkanüle und nach der Enukleation eines Auges erfolgte die Aufzeichnung der ausgelösten Impulse in Einzelfaserpräparationen des N. infraorbitalis in üblicher Weise (HENSEL 1952). Zur Erzeugung von Druckreizen wurden Testhaare verschiedener Druckfähigkeit verwandt.

Abb. 1. stellt das Verhalten einer spontantätigen mechanosensiblen Faser (grosse Impulse) und einer Temperaturfaser (kleine Impulse) bei Abkühlung und bei Erwärmung der Hautoberfläche dar. Aus der Abbildung wird ersehen, dass die Unempfindlichkeit des mechanosensiblen Receptors gegenüber thermischen Reizen sehr gross ist. Eine deutliche Frequenzabnahme kann erst oberhalb von 50° Hautoberflächentemperatur beobachtet werden. Bei dieser Temperatur werden diese Receptoren meistens irreversibel geschädigt und zeigen bei nachfolgender Abkühlung der Haut keine Tätigkeit mehr. Die grosse Empfindlichkeit des Thermoreceptors gegenüber Temperaturreizen ist aus der Abb. 1 deutlich ersichtlich.

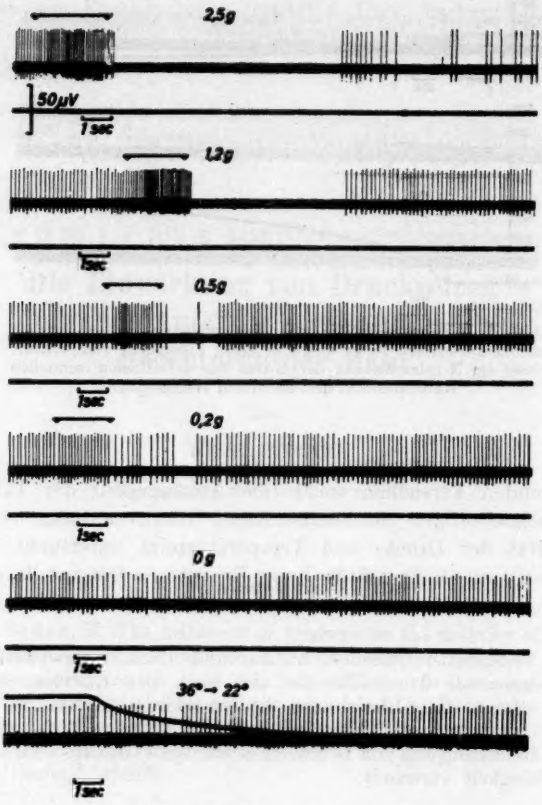


Abb. 2. Aktionspotentiale einer einzelnen mechanosensiblen Faser im N. infraorbitalis der Katze bei verschieden grossen Druckreizen und bei einem Kältesprung.

Abb. 2. stellt das Verhalten eines mechanosensiblen Receptors bei verschieden grossen Druckreizen dar. Das Receptorenfeld dieses Receptors war punktförmig. Bei zunehmender Intensität des verwandten Druckreizes kann bis zu einer gewissen Grenze eine deutliche Zunahme der Impulsfrequenz beobachtet werden. Die höchste Entladungsfrequenz dieser Faser betrug 40 Imp/sec, die durchschnittliche Entladungsfrequenz bei druckloser Haut war 8 Imp/sec. Das Nachlassen des Druckreizes führt zu einer Hem-

mung der spontanen Tätigkeit. Das impulsfreie Intervall ist von der Grösse des verwandten Druckreizes abhängig.

Diese Untersuchungen zeigen, dass in der Haut Receptoren vorkommen, die eine relativ grosse Spezifität gegenüber mechanischen Reizen zeigen. Die Funktion dieser Fasern ist bisher noch unbekannt. Die spontane Dauertätigkeit bei völlig druckloser Haut und die grosse Empfindlichkeit gegenüber mechanischen Reizen lassen uns vermuten, dass diese Receptoren Empfindungen über die Änderung des Hauttonus übermitteln. Neben den eigentlichen Druckreceptoren, die also keine spontane Tätigkeit bei druckloser Haut zeigen, übermitteln sie vielleicht auch Druckempfindungen.

## Zusammenfassung.

Im N. infraorbitalis der Katze und der Ratte können Nervenfasern spontaner Tätigkeit beobachtet werden, die eine verhältnismässig grosse Spezifität gegenüber Reizen mechanischen Charakters zeigen. Es wird vermutet, dass diese Receptoren Empfindungsqualitäten über den Hauttonus übermitteln.

## Summary.

In the infraorbital nerve of the cat and rat nerve fibres from receptors with high specific sensitivity to mechanical stimuli are demonstrated. It is believed that sensory qualities of the cutaneous tone are mediated by these spontaneously active receptors.

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Acta physiol. scand. 1959. *45*. 220—230.

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# Assay of Jorpes-Mutt Secretin and Cholecystokinin.

By

A. C. IVY and H. M. JANECEK.

Received 2 October 1958.

**Abstract.**

IVY, A. C. and H. M. JANECEK. Assay of Jorpes-Mutt secretin and cholecystokinin. *Acta physiol. scand.* 1959. *45*. 220—230. — The technique worked out in the senior author's laboratory for assaying secretin and cholecystokinin in cats and dogs has been described in detail. Highly purified samples of these hormones, supplied by the Swedish workers, were found to have a strength of 3,300 Ivy dog units of secretin and 22 Ivy dog units of cholecystokinin per mg, respectively. Due attention was paid to the extreme lability of the purified secretion.

JORPES and MUTT (1958) have produced a potent concentrate of secretin and also a mixture of cholecystokinin and pancreozymin. It was considered desirable by JORPES and MUTT and by the senior author that their secretin and cholecystokinin be assayed by the methods used in the laboratory of the senior author for the past thirty years (IVY and OLDBERG 1928, IVY *et al.* 1930, IVY, DREWYER and ORNDOFF 1930). Furthermore, the senior author has never described in detail the methods used for secretin and cholecystokinin assays.

## Assay of Secretin.

### Dog Unit of Secretin.

*Methods.*

Dogs of both sexes weighing from 10 to 16 kg were used. They had been given their last meal 24 hrs previously. They were anesthetized with sodium pentobarbital, a sufficient quantity (usually 25 mg per kg) being given intravenously to induce light surgical anesthesia. An additional single dose of 25 or 50 mg was given slowly intravenously as required to maintain a light but complete surgical anesthesia. The femoral vein was exposed for injections. The carotid artery was isolated and connected for recording the blood pressure. The abdomen was opened in the mid-line. All bleeding vessels were ligated. To facilitate the delivery of the duodenum the gastrohepatic ligament was sectioned, the duodenum rather than the pancreas being handled in all manipulations. The common bile duct was palpated and followed to its entry into the duodenum. At this point and for 1 or 1.5 cm caudally the mesenteric vessels between the pancreas and duodenum were double ligated and cut between to expose the duct of Wirsung, which was tied. (A little practice makes this a relatively non-traumatic procedure.) The duct of Wirsung, as just indicated, was found by exploring from the dorsal aspect of the duodenum. The duct of Santorini was found by exploring from the ventral aspect of the duodenum about 2.5 to 3.0 cm distal to the duct of Wirsung. It also was exposed by double ligation of the mesenteric vessels between the duodenum and pancreas. The duct was cannulated with a short-necked, only slightly beveled glass cannula, so as to have the slightly beveled opening of the cannula as far away from the bifurcation of the duct as possible. As large a cannula as possible was used. To hold the cannula in line with the duct and to avoid obstruction of the opening of the cannula by the wall of the duct, a stitch was taken into the duodenal wall and tied about the cannula anchoring it to the duodenal wall. The rubber tubing leading from the cannula to the end from which drops of juice fell for counting was 18 cm long and the inside diameter was 3 mm. The lumen of the rubber tube was filled with normal saline by a slender pipette to avoid the collection of an air-bubble in the tube.

It has been found inadvisable to use an unhealthy dog, or one that is pregnant or obese. The blood pressure should be above 150 mm Hg. If the blood pressure falls during the course of the experiment, the threshold usually increases. Sometimes the cannula becomes partially obstructed, because of mal-position or because a little blood may collect in it. In some dogs the threshold or "unit" dose remains the same for 8 or 16 hours, which is as long as we have used them. In others the "unit" dose will start to increase after 1 or 2 hours, even when the water electrolyte equivalent of the juice formed is returned.

The pancreas was, first, stimulated with a dose of secretin large enough to produce 5 or 10 ml of juice. This is what we call the "priming

**Table I.**

*Results of Secretin (EV—10) Assay in Dogs.*

| Dog No. | Body Weight | No. of Assays | Micrograms | Remarks |
|---|---|---|---|---|
| 1 | 12.3 | 5 | 1.050 | Low blood pressure |
| 2 | 10.0 | 2 | 0.175 | |
| 3 | 20.0 | 5 | 0.175 | |
| 4 | 13.2 | 4 | 0.237 | |
| 5 | 12.5 | 5 | 0.175 | |
| 6 | 12.6 | 2 | 0.300 | Low blood pressure. Pregnant. |
| 7 | 8.2 | 5 | 0.175 | |
| 8 | 16.0 | 2 | 0.150 | |
| 9 | 12.0 | 3 | 0.250 | |
| 10 | 12.2 | 4 | 0.300 | |
| 11 | 15.0 | 4 | 1.000* | Hot day (90° F) |
| 12 | 14.2 | 3 | 0.600 | Hot day (92° F) |
| 13 | 15.2 | 3 | 0.600 | Hot day (91° F) |
| 14 | 15.3 | 3 | 0.100 | |
| 15 | 7.1 | 3 | 0.200 | |
| 16 | 9.9 | 3 | 0.300 | |
| Ave.: | | | 0.380* | |

* If dogs 1 and 11 are omitted, the average is 0.295.

dose" to claer the viscous basal juice from the ducts, cannula and rubber tubing. When the flow had decreased to one drop every 2 min, assay of the unknown was started. All assays in this study were made when the rate of flow was declining but at a rate of 1 drop in 2 or 2.5 min.

When the first dose of an unknown product is to be given 0.1 mg is used, provided the product is known to be a high grade secretin concentrate. If not, 0.25 to 1.0 mg is used. After two or three dogs have been assayed, the "unit dose" is generally found to be within a range of $\pm$ 50 %, unless the animal is in "shock" or something unusual has occurred.

The preparation of secretin used for the "priming dose" should be as high grade a concentrate as one can make, since we have gained the impression that a crude secretin after stimulating tends to make the gland refractory. We have never tried to quantitatively test this impression.

*Weighing Samples:* 1.5 mg quantities of the dry powder were weighed on a balance sensitive to 0.01 mg. Dilutions were made from these samples. The dilution made from one sample was checked against other samples on the same dog. Asepsis was carefully practiced during these procedures.

*Definition of a Secretin "Dog Unit Dose":* The "dog unit" for secretin has been defined as that amount of dried material in normal saline solution which when injected intravenously during 10 or 15 sec will cause a 10 drop (0.4 ml) increase in the rate of flow during a period of

10 min, the control or basal flow not being more than one drop in two min (DENTON *et al.* 1950). The secretin product is required to be free of vasodilatin in the dose used to obtain a unit dose.

In the present study 10 drops of "secretin juice" amounted to approximately 0.5 ml. This quantity of juice was used instead of 0.4 ml and the test injection was always made when the rate of flow of pancreatic juice was 2 to 2.5 drops a minute.

## Results.

Preparation EV—10 of the secretin of JORPES and MUTT was used.

*Dog Unit:* The average "dog unit" as determined on 14 dogs amounted to 0.295 microgram with a range of 0.15 to 0.6 $\mu$g (Table I). One dog responded on one occasion to a dose of 0.10, and on another to 0.13 $\mu$g. Four dogs checked consistently at 0.175 $\mu$g during a three hour period. If the results on all of the 16 dogs are used, the mean unit dose is 0.380 and the range 0.15 to 1.0 $\mu$g.

The size of the dog unit was not correlated with the weight of the dog in the range of from 8.2 to 20 kg. Some correlation might be found if a much larger number of dogs were used, though no significant correlation was found in some 300 dogs in a previous study (DENTON *et al.* 1950).

*Deterioration of Solution of Secretin:* Doctors JORPES and MUTT informed us that bacteria rapidly destroyed their secretin in dilute solutions, and recommended the use of a sterile technique. We found this to be true. We made up all stock solutions in sterile normal saline solution in sterile glassware, using a sterile weighing bottle. The solutions were kept frozen, except when a sample was removed to make a dilution. Sterile technique was used in making the dilutions and sterile syringes were used to make the injections. Regardless of these precautions, a dilution in which 0.125 $\mu$g were present in 0.1 ml of normal saline would show detectible deterioration in 1 hour if left at room temperature.

*Blood Pressure Change:* The intravenous injection of 0.1 mg during 15 sec caused a fall of blood pressure of 50 mm Hg. An injection of 50 $\mu$g similarly occasionally caused a fall of 10 or 15 mm Hg. The injection of smaller doses had no effect on blood pressure, except that which sometimes occurs when a large vein is handled mechanically.

**Table II.**

*Results of Secretin (EV—10) Assay in Cats.*

| Cat No. | Body Weight | No. of Assays | Micrograms | Remarks |
|---|---|---|---|---|
| 1 | 3.0 | 4 | 0.100 | |
| 2 | 2.5 | 2 | 0.125 | |
| 3 | 3.1 | 5 | 0.135 | |
| 4 | 3.1 | 4 | 0.125 | Lowest 0.0625 $\mu g$ |
| 5* | 2.2 | 5 | 0.345* | Lowest 0.125* |
| 6 | 2.0 | 3 | 0.135 | Lowest 0.125 |
| 7 | 2.1 | 5 | 0.150 | |
| 8 | 2.3 | 4 | 0.106 | |
| 9 | 2.3 | 3 | 0.125 | |
| 10 | 2.2 | 3 | 0.150 | |
| Ave.: | | | 0.150* | |

* If the lowest unit dose of 0.125 is used for making the average, the average is 0.128.

## Cat Unit of Secretin.

*Methods.*

Cats of both sexes weighing from 2.0 to 3.1 kg were used. They were anesthetized with chloralose (1 %) — urethane (10 %), after preliminary etherization to the point of exposing the femoral vein for injecting the anesthetic. The abdomen was opened in the mid-line. A duct of Santorini only rarely opens into the duodenum of the cat. After making certain that none existed in the cat under experimentation, the common bile duct was found and by visual inspection and palpation it was followed to its entrance into the duodenum. (The duct of Wirsung lies adjacent to the bile duct, and usually joins it just prior to the duodenal orifice.) The pancreatic duct was carefully and partially isolated from the bile duct down to their junction. A silk thread (Size A) was passed beneath the duct with a curved surgical needle about 2 mm proximal to the junction of the ducts. The duct was cut just proximal to the junction and a cannula was inserted. (The duct took a cannula with the enlarged part just proximal to the point of the cannula measuring 1.2 to 2.0 mm. Most ducts easily took a 2.0 mm cannula.) The same rubber tubing used for the dog was used for the cat.

Since the femoral vein of the cat is much smaller than that of the dog, it may clot. A clot may cause a pooling of the injected fluid and give a false dose-response. This will occur even if the external jugular vein is used. This may be avoided by inserting a cannula into the vein, connecting it to a burette with a rubber tube, and making the injection into the rubber tube and washing it into the vein with 5 or 10 ml of normal saline from the burette.

The cat was given the "priming dose" of secretin. The test dose of secretin was given when the drops were falling at a rate of one in 2 to 2.5 min. The "*cat unit*" was defined, as in the case of the dog, 10 drops of the juice yielding a volume of 0.5 ml.

### Results.

*Cat Unit:* The average "cat unit" as determined on 10 cats was 0.15 $\mu$g with a range of from 0.1 to 0.345 $\mu$g (Table II). The minimum unit-dose observed was 0.0625 $\mu$g on one occasion in one cat.

### Discussion of the Secretin Assays.

In the dog the secretin, batch EV—10, supplied to us by Doctors JORPES and MUTT assayed approximately 3,300 uints per mg. If the results of all assays are included the value is 2,600 units per mg. The range was 1,000 to 6,600 units per mg. In one dog on one assay it amounted to 10,000 units per mg. In the cat the secretin assayed approximately 6,600 units per mg. It has been observed previously that one dog unit is equivalent to approximately 2 cat units (GREENGARD and IVY 1938, GREENGARD and STEIN 1941).

This is the most potent by weight secretin that we have ever assayed in our laboratory. The most potent we have ever produced assayed 500 dog units per mg. The most potent that has been produced consistently by us has assayed 100 dog units per mg (GERSHBEIN and KRUP 1952).

## Cholecystokinin.

### Dog Unit of Cholecystokinin.

*Methods.*

The same general methods used for the assay of secretin were used for the assay of cholecystokinin.

The method for following intragallbladder pressure changes used by IVY and OLDBERG (1928) was employed. The cystic duct was clamped and the dome of the gallbladder was cannulated to record changes in intragallbladder pressure.

In clamping the cystic duct an attempt was made to avoid the cystic artery. This was frequently not successful, but the accessory blood flow to the gallbladder is usually ample to permit an assay in most instances. A reduction of blood flow caused by clamping the artery might be expected to increase the size of the unit. Our impression is that it does, but quantitative proof has not been sought. The duct is not dissected free from the cystic blood vessels because the trauma does not appear to be justified. In the cat because of the tortuosity of the cystic duct, the latter can usually be clamped without occluding the vessels.

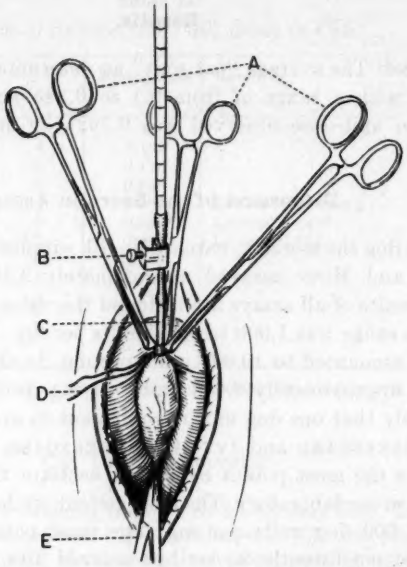


Fig. 1. Illustrating how the vulsellum forceps are used for the insertion of the trochar (C) into the gallbladder. "A", vulsellum forceps; "B", screw clamp on rubber tubing to prevent flow of bile into the glass tube serving as a manometer; "D", linen suture securing the gallbladder wall to the trochar; "E", clamp on the cystic duct.

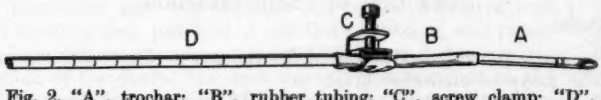


Fig. 2. "A", trochar; "B", rubber tubing; "C", screw clamp; "D", graduated glass tube serving as a manometer. The inside diameter of the system for the dog is 4 mm and for the cat 2.5 mm.

The cystic duct is exposed after sectioning the gastro-hepatic ligament by the assistant retracting the stomach and duodenum downward and to the left. The operator locates the gallbladder by placing the left hand under the liver and retracting the liver upward. When the cystic duct is located the left hand is moved inward so that the duct lies between the left index and middle finger. This is done so as not to exert pressure on the gallbladder and squeeze out bile. The cystic artery is now visualized and the duct clamped with a small hemostat avoiding the artery if possible. The dome of the gallbladder is located and its wall is grasped with two teeth of a four-toothed vulsellum forceps. The gall-

**Table III.**

*Results of Cholecystokinin Assay (V—15) in Dogs.*

| Dog No. | Body Weight | No. of Assays | Micrograms | Remarks |
|---|---|---|---|---|
| 1 | 15.0 | 2 | 100 | 20 mm Hg fall in bl. pr. |
| 2 | 12.0 | 3 | 20 | No fall in bl. pr. |
| 3 | 22.5 | 2 | 150 | 10 mm Hg fall in bl. pr. |
| 4 | 12.3 | 3 | 25 | No fall in bl. pr. |
| 5 | 17.0 | 3 | 25 | No fall in bl. pr. |
| 6 | 18.1 | 3 | 20 | No fall in bl. pr. |
| 7 | 8.5 | 1 | 25 | No fall in bl. pr. |
| 8 | 15.0 | 3 | 25 | |
| 9 | 14.2 | 3 | 40 | |
| 10 | 15.2 | 3 | 40 | |
| 11 | 14.5 | 3 | 30 | |
| Ave.: | | | 45 | |

Average volume of 10 gallbladders was 18 ml (range 8—24 ml).

bladder wall is likewise grasped about 5 mm distant with a second vulsellum forceps; then a third vulsellum is used to grasp the wall at a third point making an equilateral triangle with the first two (See Fig. 1). A linen ligature is placed so as to encircle the three forceps and is pushed beneath the unused teeth of the vulsellum forceps. The serosa of the gallbladder wall at the center of the triangle is cut (1—2 mm) to weaken the wall. The beveled point of the trochar and manometer described below is placed at the weakened point in the gallbladder wall and plunged through the mucosa with sudden short stab. The linen ligature is now tied firmly to secure the gallbladder wall about the trochar. The vulsellum forceps are now removed. The manometer is secured by an iron stand and clamp. The clamp is removed from the rubber tubing to permit bile to flow into the trochar-manometer system.

The manometer is positioned so as to minimize the respiratory excursions. The open end of the manometer may be connected with a suitable tambour if desired for making a kymographic record. Saline is added or bile withdrawn through the manometer tube so as to have an intragallbladder pressure of 7 to 10 cm of bile in the dog and 5 cm in the cat. If the manometer is connected to a tambour a tonus rhythm will frequently be observed. The tonus rhythm can also be followed by watching and recording the fluctuations of the level of bile in the manometer. The fluctuations in pressure are observed and recorded for 15 min before the cholecystokinin preparation is administered.

*The Trochar and Manometer Tubes Used for the Dog and the Cat.* The trochar and manometer system is shown in Fig. 2. The internal diameter of the trochar, rubber tubing and manometer was 4 mm in the case of the dog. In the cat, it was 2.5 mm. The trochar was 6.0 cm long in the case of the dog and 3.0 cm in the cat. The manometer was graduated in cm or 0.5 cm.

**Table IV.**

*Results of Cholecystokinin (V—15) Assay in Cats.*

| Cat No. | Body Weight | No. of Assays | Micrograms | Remarks |
|---|---|---|---|---|
| 1 | 3.1 | 1 | 25 | |
| 2 | 2.2 | 2 | 12 | |
| 3 | 2.0 | 2 | 30 | |
| 4 | 2.1 | 2 | 60* | Bilobed g. b. |
| 5 | 2.1 | 3 | 30 | |
| 6 | 1.9 | 3 | 37 | |
| 7 | 2.0 | 2 | 15 | |
| 8 | 2.3 | 4 | 17 | |
| 9 | 2.2 | 3 | 30 | |
| 10 | 2.3 | 2 | 25 | |
| Ave.: | | | 28* | |

* If bilobed g. b. is omitted, the average is 24; if evaluated at 30, the average is 25.

Average volume of 8 cats gallbladder was 2.4 ml (range 1.5—3.00 ml).

*Definition of a Cholecystokinin Dog Unit:* A dog unit of cholecystokinin has been defined as that amount of dry vasodilantin-free material (in the dose used) which when dissolved in normal saline solution and injected intravenously during 10 to 15 sec results in a more or less immediate (1 to 5 min) rise in intragallbladder pressure of 1 cm of bile.

The assay is made in the dog when the control pressure is from 7 to 10 cm of bile pressure and in the cat when the pressure is 4 or 5 cm of bile pressure. If necessary, normal saline solution (37° C) is added to bring the pressure into the indicated range. In some cases the saline may be absorbed; then, an additional amount is added.

## Results.

Preparation V—15 of cholecystokinin-pancreozymin of JORPES and MUTT was used.

*Dog Unit:* The average dog unit for cholecystokinin in 11 dogs was 45 $\mu$g, the range being from 25 to 150 $\mu$g (Table III). The smallest effective dose was 15 $\mu$g but it did not constitute a unit in that the pressure did not rise 1 cm.

It should be noted in Table III that a dose of 100 and 150 $\mu$g when given during 15 sec caused a fall in blood pressure of 10 and 20 mm/Hg. If given over a period of 3 min, a fall did not occur.

*Cat Unit:* The average cat unit for cholecystokinin for 9 cats was 28 $\mu$g, the range being from 12 to 37 $\mu$g (Table IV). On one occasion a rise of 1 cm of bile pressure (one unit) was obtained with 10 $\mu$g.

*Secretin Content of the Preparation:* This preparation (V—15) contained from one-half to one unit of secretin in each unit of cholecystokinin. Its exact secretin unitage was not assayed. It was not assayed for pancreozymin.

*Deterioration:* The cholecystokinin-pancreozymin preparation in a dilution of 20 μg/ml did not deteriorate over a period of 1 or 2 hours at room temperature when perpared and handled under sterile conditions.

### Discussion of Cholecystokinin Assays.

The cholecystokinin assayed an average of 22 dog units and 35 cat units/mg. This is the most potent cholecystokinin we have assayed in our laboratory. We have been able to make a product consistently which assayed 6 units/mg (DENTON, GERSHBEIN and IVY 1950), though occasionally a product assaying 10 units/mg has been obtained.

The presence of secretin in the preparation V—15 should be advantageous for clinical use. It promotes a flow of pancreatic juice which resists biliary-pancreatic reflux and simulates physiological conditions.

The vasodilator which exists in preparation V—15 should cause only facial flush in some patients if the preparation is given slowly.

It is realized that the method employed for registering intragallbladder pressure may not be ideal. However, we preferred not to use an isometric method since under normal conditions the contraction of the gallbladder is not isometric.

Since we did not use an isometric method, difficulties arise in comparing cat units with dog units. The diameter of the lumen of the trochar manometer system in the dog was 4 mm and in the cat 2.5 mm. The average volume of the gallbladder of the dog was 18 ml and of the cats 2.4 ml. This yields approximately a cat to dog gallbladder volume ratio of 1 to 8. When 1 cm of rise in pressure is used the ratio of the volume displaced from the gallbladder into the manometer in the dog is 1 to 166 and in the cat 1 to 50. To obtain in the cat the same volumetric displacement the diameter of the trochar manometer system should be 1.4 mm instead of 2.5 mm. However, a method for making a physiologically valid comparison of cat and dog units will have to be determined experimentally.

## Summary.

1. As determined in 16 dogs and 10 cats the preparation EV—10 of JORPES and MUTT contains an average of 3,300 IVY dog units and 6,600 IVY cat units of secretin per mg.

2. As determined in 11 dogs and 10 cats, the preparation V—15 of cholecystokinin-pancreozymin-secretin of JORPES and MUTT contains an average of 22 IVY dog units and 35 Ivy cat units of cholecystokinin per mg.

3. These are the most potent preparations of secretin and cholecystokinin ever assayed in our laboratory.

We desire to thank Mr. Norman S. Litowitz who assisted us with some of the assays.

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Acta physiol. scand. 1959. *45*. 231—237.

From the Department of Physiology, University of Turku, Finland.

# Studies of the Effect of X-Rays on the Glucuronide Synthesis by the Liver.

By

K. HARTIALA, V. NÄNTÖ and U. K. RINNE.

Received 22 October 1958.

**Abstract.**

HARTIALA, K, V. NÄNTÖ, and U. K. RINNE. Studies of the effect of X-rays on the glucuronide synthesis by the liver. *Acta physiol. scand.* 1959. *45*. 231—237. — The effect of local X-irradiation of the liver on the hepatic glucuronide synthesis capacity was studied in rats. The liver was irradiated under nembutal anesthesia. The glucuronide synthesis of the liver slices was then measured *in vitro* using o-aminophenol as the toxic substance. The 1,200 r radiation dose caused a depression in the glucuronide conjugation capacity, this effect reached its maximum on the 3rd day after the irradiation. This was followed by a compensatory phase. When applying a smaller radiation dose (400 r) an increase in the conjugation capacity was noted on the first day. This was followed by a decrease slightly below the control level and later by a compensatory phase. The results are discussed in light of our previous similar studies with rat gastric mucous membrane.

Several investigations are at hand describing the effect of ionizing radiation, particularly the biochemicals effects of X-rays, on the liver tissue (recent reviews on the subject by ELLINGER 1945, KAY and ENTENMAN 1956, HOLMES 1956, SMITH and LOW-BEER 1957).

As is well known the liver performs the detoxication of harmful substances and compounds a. o. by means of glucuronide conjugation (*e. g.* WILLIAMS 1947). Similar glucuronide synthesis has been shown to take place also in the mucous membrane of the gastrointestinal tract (HARTIALA 1954). We have recently reported our observations on the effect of X-rays on the glucuronide synthesis carried by the gastric mucous membrane in rat (HARTIALA, NÄNTÖ and RINNE 1958). In the present work we have studied this effect in the hepatic tissue.

## Material and Methods.

Altogether 110 male rats were used ((Wistar, weight 180—220 g). One of the hepatic lobes was exposed under nembutal anesthesia through a mid-line excision and covered with a piece of gauze moistened with saline. The other parts of the animal were protected with a 3 mm thick lead shield. The irradiation of the isolated liver lobe was then performed with a 180 kV X-ray machine (10 mA, 0.5 cm Cu). One group of the animals (Group A, 45 animals) received a 400 r radiation dose, the other group (B, 46 animals) was given 1,200 r. After being irradiated, the liver lobe was replaced in the abdominal cavity and the wall closed with silk sutures. Prior and after this procedure the animals were fed with their usually laboratory diet and water ad libitum. The capacity of the hepatic glucuronide synthesis was measured 1/2, 1, 3, 5, 8 and 12 days after the irradiation. This was performed in vitro by using o-aminophenol as the toxic agent according to the previously described procedures (LEVVY and STOREY 1949, HARTIALA and RONTU 1955). Control analyses on 19 animals were carried out in the same connection.

## Results.

The results are listed in Table I and they are expressed as $\mu$g o-aminophenol glucuronide produced by the liver parenchyma per 100 mg dry weight tissue. Fig. 1 illustrates these results in comparison to the corresponding control values at various phases. It appears that at the beginning the 400 r irradiation dose is followed by a stimulation of the glucuronide synthesis. The difference from the control values on the 1st day after the irradiation is significant, $P < 0.05$. The corresponding increase in the 1,200 r group is not significant. After 3 days a decrease in the synthetic capacity is noted in both groups. In the 400 r group it reaches the control level whereas it in the 1,200 r group

**Table I.**

*The results expressed as μg of o-aminophenolglucuronide produced by the liver parenchyma per 100 mg dry weight tissue. (P values refer to comparison with the control animals.)*

| Time after irradiation | Group A (400 r) | | | Group B (1,200 r) | | |
|---|---|---|---|---|---|---|
| | Number of animals | μg/100 mg | P | Number of animals | μg/100 mg | P |
| 12 hours ..... | 8 | 152 ± 14.2 | >0.05 | 8 | 152 ± 18.2 | >0.05 |
| 1 day ..... | 8 | 190 ± 23.5 | <0.05 | 8 | 157 ± 18.2 | >0.05 |
| 3 days ..... | 7 | 127 ± 6.8 | >0.05 | 7 | 65 ± 6.2 | <0.001 |
| 5 » ..... | 8 | 169 ± 12.1 | >0.05 | 7 | 120 ± 4.0 | >0.05 |
| 8 » ..... | 8 | 172 ± 19.4 | >0.05 | 8 | 165 ± 14.8 | >0.05 |
| 12 » ..... | 6 | 160 ± 12.6 | >0.05 | 8 | 148 ± 17.1 | >0.05 |
| Control animals | 19 | 142 ± 9.4 | | | | |

is significantly below the control level ($P < 0.001$). The difference between the two groups is also highly significant at this phase ($P < 0.001$). This phase is followed by a compensatory one; the detoxication capacity shows a tendency to rise above the control level although this is not statistically significant in either groups. After 12 days has the detoxication capacity in both groups returned nearly to the control level. The difference between the two groups was significant both on the 3rd and 5th day ($P < 0.001$ and $P < 0.01$) whereas no difference was evident at the remaining points.

## Discussion.

These results indicate that the X-rays have a similar depressive effect on the liver glucuronide synthesis as was noted in the gastric mucosa (HARTIALA, NÄNTÖ and RINNE 1958). The reduction was however less in the liver which is in accordance with the available information about the radiosensitivity of the liver. The hepatic glucuronide synthesis is not much affected by the smaller 400 r irradiation dose whereas the effect on the gastric mucosa is at its maximum point (5 days after the irradiation) equal with the both radiation doses.

Also the recovery of the liver after the 1,200 r dose appears to

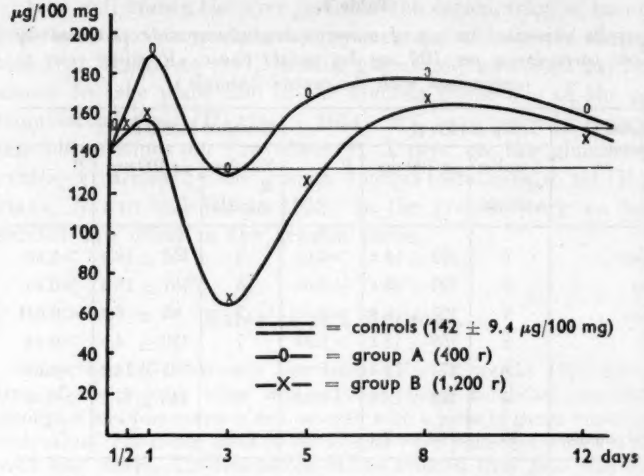


Fig. 1. Effect of X-ray irradiation on the o-aminophenolglucuronide synthesis by the liver parenchyma. Results expressed as µg o-aminophenolglucuronide per 100 mg dry weight liver tissue.

be rapid and on the 8th day period there seems to be the tendency for over-compensation. The glucuronide synthesis of the gastric mucosa is far more impaired and the recovery from the 1,200 r dose occurs only after 21—32 days. The hepatic tissue has been considered to be rather resistant towards radiation effects, this opinion been based mainly on the slight histological changes detected (WARREN and FRIEDMAN 1942, RHOADES 1948). In several studies biochemical changes have been noted following X-rays (HEEREN and PANSDORF 1931, DRUCKREY, LOW-BEER and REISS 1932, WARREN and WHIPPLE 1922, ABDERHALDEN 1940, DUBOIS and PETERSEN 1954, KUNKEL and PHILLIPS 1952, MCKEE and BRIN 1956, KAY and ENTENMAN 1956, SMITH and LOW-BEER 1957). Energy-rich phosphate compounds have been demonstrated to participate in the hepatic glucuronide synthesis. The oxidation of uridinediphosphoglucose to uridinediphosphoglucuronic acid is carried by specific dehydrogenase-enzymes (DUTTON and STOREY 1954, 1955, SMITH and MILLS 1954). Those enzyme systems which carry on the final conjugation but being most likely in the microsomes of the liver cells, have not yet been identified. According to some investigators $\beta$-glucuronidase enzymes

should participate in the process (FISHMAN 1940, FISHMAN and GREEN 1956, FISHMAN and SIE 1957). Not much information is available as to the effect of radiation on these enzyme systems (ORD and STOCKEN 1953).

Histological changes appearing soon after the irradiation have later been shown (WARREN, HOLT and SOMMERS 1952). CASATI and CAFISSI (1937) irradiated livers taken from freshly killed rats in order to eliminate the effect of regeneration on the results and noted "very severe protoplasmic changes". It has been assumed that the markedly great regeneration power possessed by the liver cells contribute for a great part to the difficulties in promotion of histological changes comparable to the physiological and biochemical changes following irradiation (WARREN and FRIEDMAN 1942). In view of the glandular structure of the liver and its "high metabolic rate" one could expect this organ to be relatively radiosensitive (ELLINGER 1945). It is to be noted, however, that a great part of the detected biochemical changes might be indirect or secondary of nature since it has not always been shown that the effect of the radiation is only applied to the liver (whole body irradiation is often used). When comparing the present results obtained with the liver with those obtained after local X-irradiation of the gastric mucous membrane (HARTIALA, NÄNTÖ and RINNE 1958) we find that the effect on the glucuronide synthesis are principally the same in both cases. Nevertheless the hepatic tissue appears to be far more resistant and the recovery takes place also very rapidly. We have not been able to note in our later additional experiments with gastric mucosa a similar increase in the glucuronide conjugation capacity as is noted in the liver on the first day after the irradiation of the organ with the 400 r dose. This is in some way in agreement with the observations of IVY *et al.* (1923) who claim that small X-ray doses do not stimulate the excretory function of gastric mucosa. Since the gastric mucosa evidently is very radiosensitive it might still be possible that by applying small doses enough a similar increase could be obtained.

## Summary.

The effect of local X-irradiation of the liver on the hepatic glucuronide synthesis capacity was studied in rats. This was measured in vitro using o-aminophenol as the toxic substance.

It was found that the 1,200 r radiation dose caused a depression in the glucuronide conjugation capacity, this effect reached its maximum on the 3rd day after the irradiation. This was rapidly followed by a compensatory phase. When applying a smaller radiation dose (400 r) an increase in the conjugation capacity was noted on the first day. This was followed by a decrease slightly below the control level and later by a compensatory phase. The results are compared with the previous observations made on the effect of X-irradiation on the glucuronide conjugation capacity of rat gastric mucous membrane.

This study was supported by The SIGRID JUSELIUS Stiftelse.

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Acta physiol. scand. 1959. 45. 238—254.

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# Quantitative Determination of Cholinesterase in Individual Spinal Ganglion Cells.

By

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Received 31 October 1958.

**Abstract.**

GIACOBINI, E. Quantitative determination of cholinesterase in individual spinal ganglion cells. *Acta physiol. scand.* 1959. *45.* 238—254. — The cholinesterase content of neurones belonging to the spinal ganglia of the rat has been investigated microchemically by means of a Cartesian diver technique. Most of the cells examined showed a measurable ChE activity. The ChE activity varies significantly from cell to cell and the statistical analysis of the data showed that two different groups of cells are present having respectively high and low activity. An additional group of cells did not show any measurable activity. The results are discussed in the light of morphological and physiological data.

In recent histochemical work on isolated spinal ganglion cells of frog and rat, it has been demonstrated (GIACOBINI 1956) by means of a modified thiocholine method that two types of cells could be distinguished with respect to their AChE[1] activity. About

[1] Abbreviations: ChE (cholinesterase) = all types of enzyme splitting choline esters irrespective of their specificity for certain substrates; AChE = acetylcholinesterase, true cholinesterase, specific cholinesterase, acetocholinesterase, "e" type (e = erythrocytes), cholinesterase I. Non spec. ChE = non specific cholinesterase, pseudo cholinesterase, "s" type (s = serum), butyro- and propiono cholinesterase, cholinesterase II.

85—90 % of the total cell population exhibited slight or no AChE activity after short incubation periods (15 min) while 10—15 % showed high activity. The crystals of copper thiocholine sulphate produced by the histochemical reaction were distributed all over the surface of the cell body and the axon and in some cases could also be seen within the cytoplasm. The nucleus was apparently devoid of any AChE activity. The cells having high activity and those with a low activity were randomly distributed within the ganglion and there was no relation between different morphological types of cells and their AChE activity. By employing a specific substrate (BuThCh)[1] and selective inhibitors it was possible to demonstrate non specific ChE in some cells.

In the present investigation the ChE activity has been determined in the cell body and in the proximal segment of the neurite of single spinal ganglion cells by means of a microgasometric method, the Cartesian diver technique (Linderström-Lang 1937, Zeuthen 1953, Zajicek and Zeuthen 1956).

## Material and Methods.

The sixth, seventh and eighth cervical spinal ganglia of rats, weighing between 150 and 200 g were used in all experiments. After decapitation of the animal the spinal ganglia were dissected out and put into Ringer's solution. The isolation of the single nerve cells and axons, and the quantitative determination of their AChE activity by means of the Cartesian diver technique, is the same which has previously been employed (Giacobini and Zajicek 1956, Giacobini 1957, Giacobini and Holmstedt 1958) and will therefore only be briefly dealt with here.

A single cell with a segment of its axon was transferred by means of a micropipette to a drop of paraffin oil on a microscope slide and inspected in phase contrast under high magnification. Spinal ganglion cell bodies and axons could easily be isolated from their surrounding capsule cells and from glial processes (see Fig. 1—2). In some cases a segment of the proximal part of the neurite, measuring about 100 $\mu$ in length, was cut off and transferred to another drop of paraffin oil. The maximum and minimum diameters of the cell and of the axon were recorded by means of a micrometer eyepiece (Baker) at 450 ×. The volume of the cell body was determined from the computed diameter, $\sqrt{d^1 \times d^2}$, where $d^1$ and $d^2$ are the maximum and minimum diameters of the cell, by the formula for a sphere. The volume of the axon segments were determined from the diameter of the axon and its length by the formula for a cylinder.

[1] BuThCh = butyrylthiocholine.

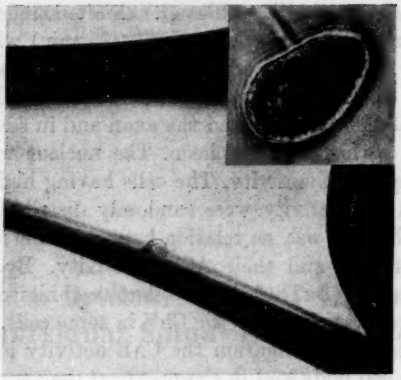

Fig. 1. A spinal ganglion cell (max. diam. about 40 $\mu$) of rat placed in the diver is shown at two different magnifications.

The structure to be analyzed was sucked into a diver having a gas volume of about 0.2 $\mu$l and weighing about 0.8 mg (see Fig. 1). In some experiments smaller divers were employed having gas volumes of 0.01—0.08 $\mu$l and weighing 10—100 $\mu$g. The glass employed was chemically stable "Phoenix workable heat resisting glass" and the diver was made partly by hand and partly with the help of a microforge according to the technique described by GIACOBINI (1959).

The sensitivity of the method was $10^{-4}$ $\mu$l $CO_2$ or $10^{-5}$ $\mu$l $CO_2$/hour depending on the dimensions of the diver employed. The accuracy of the measurements was estimated to 5 %. The substrate employed was acetylthiocholine (AThCh, acetylthiocholine iodide Hoffman La Roche $6.5 \times 10^{-3}$ M).

Regarding the morphology of the cells employed in this investigation it should be pointed out that in the rat, the spinal ganglion cells are unipolar, approximately spherical in shape and have a highly viscous cytoplasm. The cervical spinal ganglia contain the largest cells and the greatest number of cells (10—12,000) of any spinal ganglion (HATAI 1902—07) and the cells can be classified according to size into two groups having diameters varying from 20—30 $\mu$ (40 %) and 30—60 $\mu$ (60 %) respectively.

In agreement with previous investigations (a. o. see DEITERS 1865, DONALDSON 1897, LEVI 1905, CAJAL 1909—11, HÄGGQVIST 1938) in which mostly fixed material has been employed, a certain direct correlation was seen between the fiber size and the diameter of the cell from which the fiber originates.

In the mammal spinal ganglia two types of cells have been described by most authors (see a. o. v. LENHOSSEK 1886, FLEMING 1895, COX 1898,

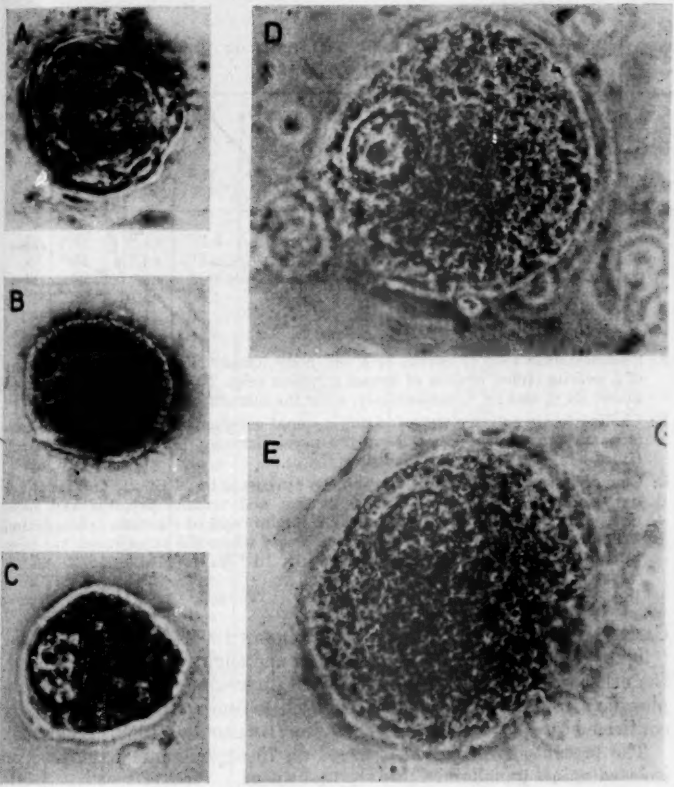


Fig. 2. Spinal ganglion cells from rat.

A: Note the thick layer of capsule cells around the isolated cell body.

B: AChE activity after a short incubation (10 min). Small needle-shaped crystals sticking out from the cytoplasm are visible. (See text.)

C: Isolated cell body in Ringer-AThCh solution.

D and E: Two cell bodies after 48 h. permanence in a Ringer solution at room temperature. The nucleus and nucleolus together with other morphological details of the cell are still visible. In E) the neurite has been stripped out and the cell has broken at the axon hillock. In spite of this the cytoplasmic structure is still well maintained.

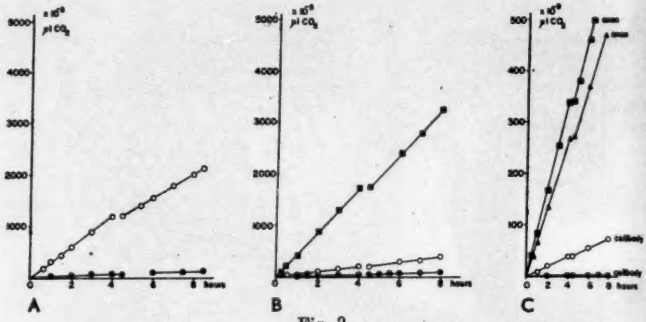


Fig. 3.

A: Cholinesterase activity curves of a cell body (empty circles) and of a segment of a neurite (filled circles) of spinal ganglion cells. The activity is reduced to about 93 % and 82 % respectively, after the introduction of the inhibitor.

B: Cholinesterase activity curves of single spinal ganglion cells. Two of these cells (squares and empty circles) have the same diameter (36 $\mu$). The third cell (filled circles) has greater dimensions (58 $\mu$).

C: Comparative representation of the activity curves in $\mu$l $CO_2 \times 10^{-3}$/hour per unit surface (triangles and empty circles) and volume (squares and filled circles) of a cell body (empty and filled circles) and of its axon (squares and triangles). The intervals in the curves indicate where the experiment has been interrupted for the addition of Mipafox $5 \times 10^{-4}$ M.

CAJAL 1898—1906, HATAI 1901—07, RANSON 1908—12). The first type ("light cells") being unipolar with a big and slightly staining cell body, the other type ("dark cells") with small dimensions and a strong cytoplasmic affinity for staining reagents. These findings have been recently confirmed by electron microscopy studies (BEAMS *et al.* 1952, HESS 1955).

The present investigation is based on 75 experiments performed on isolated spinal ganglion cell bodies and axons.

## Results.

### *A) ChE activity of individual spinal ganglion cells.*

The ChE activity varied greatly from one cell to another, this variation having no direct relation to the dimensions of the cells. In order to illustrate this fact three cells have been selected and the values of their activities have been presented in Fig. 3 B. Two of these cells had approximately the same diameter (36 $\mu$) but one of them (squares) had an activity of $4.2 \times 10^{-3}$ $\mu$l $CO_2$/hour while the activity of the other (empty circles) was about ten times lower ($0.41 \times 10^{-3}$ $CO_2$ $\mu$l/hour). The third cell (filled

**Table I a.**

*Cholinesterase activity of single nerve cell bodies from spinal ganglia of rat.*[1, 2]

| Diver no. | Computed diam. in $\mu$ | Cell surface in $\mu^2$ ($\times 10^3$) | Cell volume in $\mu^3$ ($\times 10^3$) | Activity/unit area $\mu$l $CO_2/\mu^2$/h. ($\times 10^{-7}$) | Activity/unit volume $\mu$l $CO_2/\mu^3$/h. ($\times 10^{-9}$) | Total activity $\mu$l $CO_2$/cell/h. ($\times 10^{-3}$) |
|---|---|---|---|---|---|---|
| 42 | 52 | 8.45 | 73.6 | 0.536 | 6.160 | 0.453 |
| 43 | 35 | 3.90 | 22.3 | 2.060 | 3.600 | + 0.800 |
| 44 | 33 | 3.38 | 18.7 | 1.384 | 25.00 | 0.468 |
| 51 | 57 | 10.4 | 96.5 | 0.330 | 3.470 | 0.334 |
| 56 | 49 | 7.25 | 60.7 | 0.174 | 2.040 | 0.126 |
| 59 | 47 | 6.70 | 54.0 | 0.360 | 4.470 | 0.242 |
| 63 | 47 | 6.70 | 54.0 | 0.220 | 2.740 | 0.133 |
| 64 | 58 | 10.4 | 102 | 0.105 | 1.080 | + 0.111 |
| 66 | 47 | 6.70 | 54.0 | 0.296 | 3.680 | 0.198 |
| 67 | 58 | 10.4 | 102 | 0.528 | 5.400 | 0.550 |
| 68 | 40 | 5.0 | 57.3 | 0.660 | 9.780 | 0.330 |
| 72 | 30 | 2.80 | 14.1 | 2.320 | 46.00 | 0.650 |
| 73 | 48 | 7.24 | 58.0 | 0.688 | 8.650 | 0.495 |
| 74 | 30 | 2.80 | 14.1 | 1.872 | 37.12 | 0.525 |
| 75 | 36 | 2.53 | 24.5 | 1.032 | 16.96 | + 0.415 |
| 78 | 27 | 2.27 | 10.2 | 3.460 | 16.80 | 0.787 |
| 79 | 58 | 10.4 | 102 | 0.100 | 1.030 | + 0.105 |
| 80 | 26 | 2.10 | 9.15 | 4.000 | 92.00 | 0.840 |
| 81 | 34 | 3.60 | 20.5 | 1.772 | 30.72 | 0.630 |
| 82 | 25 | 1.95 | 8.20 | 3.480 | 82.40 | + 0.680 |
| 84 | 45 | 6.30 | 48.0 | 0.517 | 6.800 | 0.325 |
| 85 | 45 | 6.30 | 48.0 | 0.595 | 7.856 | 0.375 |
| 86 | 55 | 9.45 | 86.5 | 0.350 | 3.840 | + 0.332 |
| 88 | 45 | 6.30 | 48.0 | 0.600 | 8.880 | 0.375 |
| 89 | 55 | 9.45 | 86.5 | 0.092 | 1.000 | 0.087 |
| 90 | 54 | 9.07 | 82.0 | 0.136 | 15.28 | 0.125 |
| 101 | 45 | 6.30 | 84.0 | 0.446 | 5.888 | 0.278 |
| 102 | 45 | 6.30 | 84.0 | 0.320 | 4.224 | 0.202 |
| | | | | $M_1 = 1.02 \pm 0.207 \times 10^{-7}$ $\delta_1 = 1.10$ | $M_1 = 16.1 \pm 4.37 \times 10^{-9}$ $\delta_1 = 22.108$ | $M_1 = 0.38 \pm 0.039 \times 10^{-3}$ $\delta_1 = 0.212$ |

[1, 2] See page 244.

circles) presented in Fig. 3 B was the greatest of the three cells but had the lowest activity (respectively 3.7 or 37 times lower than that of the previous cells).

The intervals in the curves indicate where the experiment has been interrupted for the addition of Mipafox $5 \times 10^{-6}$ M. This concentration of Mipafox gives a complete inhibition of non spec. ChE under the experimental conditions described (GIACOBINI and ZAJICEK 1956, GIACOBINI 1959).

**Table I b.**

*Cholinesterase activity of single nerve cell bodies from spinal ganglia of rat.*

| Diver nr. | Computed diam. in $\mu$ | Cell surface in $\mu^2$ ($\times 10^3$) | Cell volume in $\mu^3$ ($\times 10^3$) | Activity/unit area $\mu$l $CO_2/\mu^2$/h. ($\times 10^{-7}$) | Activity/unit volume $\mu$l $CO_2/\mu^3$/h. ($\times 10^{-8}$) | Total activity $\mu$l $CO_2/\mu^3$/h. ($\times 10^{-3}$) |
|---|---|---|---|---|---|---|
| 53 | 42 | 5.5 | 57.0 | 5.280 | 50.40 | + 2.91 |
| 55 | 52 | 8.7 | 73.0 | 2.200 | 26.10 | 1.92 |
| 71 | 59 | 10.9 | 107 | 1.472 | 13.76 | 1.47 |
| 103 | 36 | 4.1 | 24.0 | 10.24 | 172.0 | + 4.20 |
| 104 | 38 | 4.5 | 29.0 | 5.920 | 101.6 | 2.94 |
| | | | | $M_2 = 5.14 \pm 1.59 \times 10^{-7}$ $\delta_2 = 3.55$ | $M_1 = 72.8 \pm 28.9 \times 10^{-8}$ $\delta_2 = 64.8$ | $M_2 = 2.69 \pm 0.47 \times 10^{-3}$ $\delta_2 = 1.05$ |
| 52 | 55 | — | — | | no meas. activity | |
| 54 | 48 | — | — | | no meas. activity | |
| 57 | 35 | — | — | | no meas. activity | |
| 58 | 36 | — | — | | no meas. activity | |
| 61 | 60 | — | — | | no meas. activity | |
| 62 | 40 | — | — | | no meas. activity | |
| 65 | 34 | — | — | | no meas. activity | |
| 69 | 38 | — | — | | no meas. activity | |
| 70 | 45 | — | — | | no meas. activity | |
| 77 | 48 | — | — | | no meas. activity | |
| 83 | 48 | — | — | | no meas. activity | |
| 105 | 34 | — | — | | no meas. activity | |
| 106 | 55 | — | — | | no meas. activity | |
| 107 | 58 | — | — | | no meas. activity | |
| 108 | 55 | — | — | | no meas. activity | |
| 109 | 58 | — | — | | no meas. activity | |
| 110 | 60 | — | — | | no meas. activity | |

[1] In Table I a, I b, II and III the sign + precedes a figure expressing predominantly AChE activity or AChE activity alone.

[2] The data were computed adopting the following statistical method: the standard deviation was calculated according to $\delta = \sqrt{\frac{\varepsilon a^2}{n-1}}$ where $a$ is the deviation from the mean value and n the number of observations; e (M) the mean error of the arithmetical mean was calculated with the formula: $e(M) = \pm \frac{\delta}{\sqrt{n}}$.

The identity in the slopes of the curves before and after the gap shows that the splitting of acetylthiocholine continues at the same rate after the addition of the inhibitor. Some cells, however, as demonstrated in Fig. 3 A, showed a certain reduction of activity after the inhibitor had been added. The curves in this figure show that in the cell body (empty circles) the activity is reduced to about 93.2 % and in the neurite (filled circles) to 82 % of the orig-

inal value, demonstrating a low non spec. ChE activity. The presence of non spec. ChE in the spinal ganglion cells could be confirmed by using a specific substrate (BuCh, $5 \times 10^{-6}$ M) together with a specific inhibitor (BW 284 C 51).

Fig. 3 C is a comparative representation of the activity curves in $\mu$l $CO_2 \times 10^{-2}$ per surface (triangles and empty circles) and per volume (squares and filled circles) unit of the cell body and its neurite. The figure demonstrates that in a spinal ganglion cell the activity per unit of surface or volume of the neurite is several times higher than that of the cell body, in this case the ratio being 6.7 (surface) and 82.2 (volume) respectively (see also Table III, diver no. 79 and 93 a).

Table III shows the ChE activity of the cell bodies and axons belonging to the same cell. The values are reported according to a progressive order following the cell dimensions. Comparing the activity per unit of surface or volume of the cell body with that of its axon the latter is found to be higher: the ratio between them lying between 0.692 and 10.40 (surface) and between 8.70 and 131.15 for the volume.

A direct correlation between the activity of the cell body and that of the axon in the same cell becomes also evident by the examination of this table; nevertheless, the ratio axon/body activity is higher in those cells which have less activity in their bodies.

As shown in Table III the lack of ChE activity in the cell body is always accompanied by lack of activity in the axon; this fact is in agreement with that previously found in the sympathetic ganglion cells of frog and rat (GIACOBINI 1957).

In some experiments, a single cell body was incubated during 10—15 min in the histochemical medium before the ChE activity was determined with the diver apparatus.

Fig. 2 B shows a single cell preparation (comp. diam. 36 $\mu$) in which the ChE activity is demonstrated by small needle-shaped crystals of copper thiocholine sulphate sticking out from the cytoplasm.

The ChE activity of this cell body, subsequently determined in the diver, could be estimated to $0.4 \times 10^{-3}$ $\mu$l $CO_2$/hour (diver no. 75, Table I a).

*B) ChE activity of single cell bodies.*

The total ChE activity of 50 single cell bodies expressed in $\mu$l/$CO_2$/hour is reported in Table I a and I b together with the

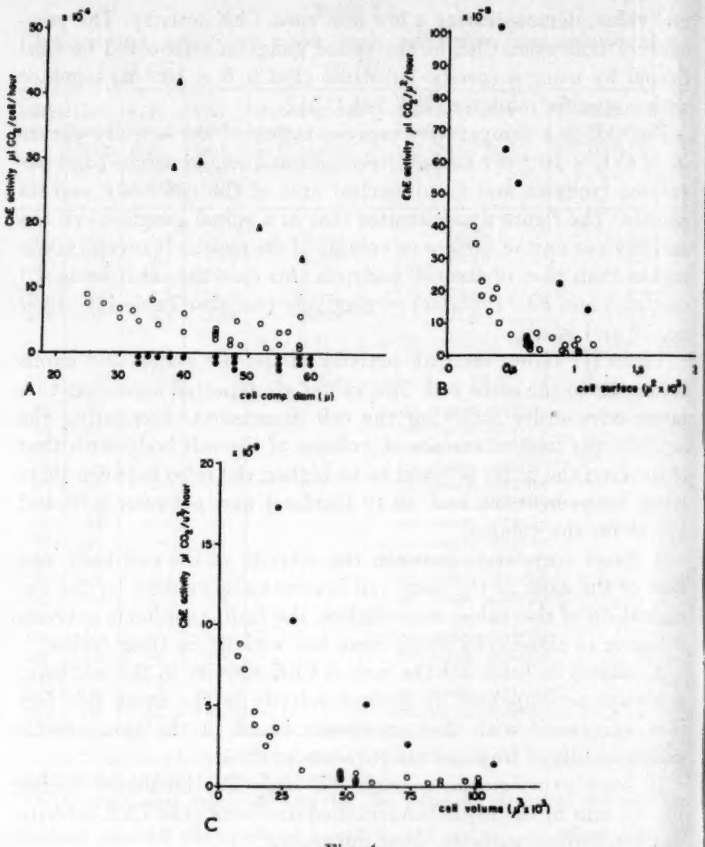


Fig. 4.

A: Total cholinesterase activity of 50 single cell bodies of spinal ganglion cells of rat, plotted against the cell computed diameter. The graph shows that in respect of their ChE activity the cell bodies can be divided in two groups having:

1) high activity (triangles) and low activity (empty circles) and

2) no measurable activity (filled circles).

B and C: The activity per unit surface (B) or volume (C) is plotted against the cell surface or the cell volume. It can be seen that the two groups of cells showing different ChE activity are still maintained.

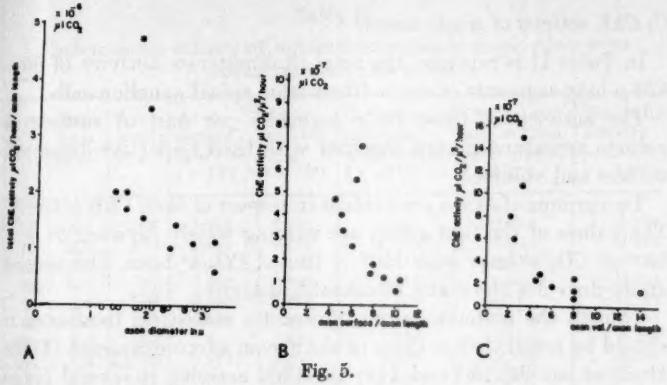


Fig. 5.

A: The total cholinesterase activity of 65—420 $\mu$ long segments of single fibers from spinal ganglion cells is plotted against the axon length in $\mu$.

B: The cholinesterase activity per unit of surface is plotted against the ratio axon surface/axon length.

C: The cholinesterase activity per unit of volume is plotted against the ratio axon volume/axon length.

activity per unit of volume and of surface. The computed diameter, the volume and the surface of the cell are also reported.

The tables show a wide variation of the ChE activity of the different cell bodies (between $0.087 \times 10^{-3}$ $\mu$l $CO_2$/hour and $4.2 \times 10^{-3}$ $\mu$l $CO_2$/hour). In respect to their ChE activity the cell bodies can be divided into two different groups having a high activity (mean value of the total activity $2.69 \pm 0.47 \times 10^{-3}$ $\mu$l $CO_2$/hour) and a low activity (mean value of the total activity $0.38 \pm 0.039 \times 10^{-3}$ $\mu$l $CO_2$/hour) or no measurable activity (lower than $1 \times 10^{-6}$ $\mu$l $CO_2$/hour).

The proportion of the two groups in the material investigated is 66 % (10 % high activ., 56 % low activ.) and 34 % (no activ.) respectively.

The two groups of cells are demonstrated graphically in Fig. 4 A where their activity is plotted against the diameter, and in Fig. 4 B and C where the activity is expressed in unit of surface or of volume. An examination of the graphs shows that the values in Fig. 4 B and 4 C show a tendency to fall along two different curves of hyperbolic form.

In Fig. 4 A, the group with higher activity values has been represented with triangles and in Fig. 4 B and 4 C with filled circles.

*C) ChE activity of single axons.*

In Table II is reported the total cholinesterase activity of 65—420 $\mu$ long segments of single fibers from spinal ganglion cells.

The activity of these fiber segments per unit of surface or volume are also reported together with their respective diameter, surface and volume.

Two groups of axons are evident in respect of their ChE activity. The values of the first group are varying widely between $84.6 \times 10^{-9}$ $\mu$l $CO_2/\mu^3$/hour and $1507 \times 10^{-9}$ $\mu$l $CO_2/\mu^3$/hour. The second group does not show any measurable activity.

Though the material is too limited for statistical treatment it should be noted that in three of the eleven axons examined (Table II, diver no. 94, 100 and 119) the ChE activity is several times higher than in the other. When the values of Table II are graphically reported in Fig. 5 A, 5 B and 5 C they showed a tendency to give origin to a curve analogue to those reported for the cell bodies. The second curve is not clearly discernible for lack of values.

An inverse relation of the activity to the diameter, surface and volume unit is also seen in analogy with that previously demonstrated for the cell bodies. In some axons a low non spec. ChE activity can be demonstrated (see Fig. 3 A) but most of them showed AChE activity alone.

## Discussion and Conclusion.

In general it can be said that a good correlation is found between the results of the previous histochemical investigation (GIACOBINI 1956) and those obtained by means of the micromanometric technique employed in this work, but, further details on the peculiar distribution of the ChE in these neurones could only be demonstrated by means of the second method.

Earlier histochemical work on sections of spinal ganglia of different mammals (KOELLE 1951—1955) demonstrated that primary sensory neurones exhibit uniformly little or no AChE activity. More recently (1955) however, the same author incubating the sections for longer periods (two hours) has succeeded in demonstrating moderate and variable staining intensity in the ganglion cells of rat, rabbit and rhesus monkey. It should however be emphasized that during such a prolonged incubation period grave diffusion artifacts are produced which make the histochemical picture

**Table II.**

*Cholinesterase activity of segments belonging to single fibres from spinal ganglion cells of rat.*

| Diver no. | Axon diam. in $\mu$ | Axon length in $\mu$ | Axon surface in $\mu^2$ ($\times 10^3$) | Axon volume in $\mu^3$ ($\times 10^3$) | ChE activity/ unit area $\mu$l $CO_2/\mu^2$/h. ($\times 10^{-7}$) | ChE activity/ unit volume $\mu$l $CO_2/\mu$l/h. ($\times 10^{-5}$) | Total ChE activity $\mu$l $CO_2$/h. ($\times 10^{-3}$) |
|---|---|---|---|---|---|---|---|
| 87 | 3.2 | 110 | 1.140 | 0.885 | 1.100 | 142.5 | 0.126 |
| 91 | 2.8 | 120 | 1.060 | 0.728 | 1.245 | 181.5 | 0.132 |
| 92 | 2.5 | 100 | 0.785 | 0.490 | 1.810 | 290.0 | 0.142 |
| 93a | 3.2 | 80 | 0.803 | 0.645 | 0.679 | 84.60 | + 0.054 |
| 94 | 2.0 | 110 | 0.690 | 0.346 | 5.560 | 1077 | + 0.384 |
| 96 | 1.9 | 100 | 0.596 | 0.254 | 2.590 | 607.0 | 0.154 |
| 100 | 2.0 | 420 | 2.820 | 1.320 | 7.060 | 1507 | + 1.990 |
| 106 | 1.6 | 95 | 0.477 | 0.763 | 4.000 | 250.0 | 0.191 |
| 109 | 2.4 | 85 | 0.641 | 0.385 | 1.432 | 239.0 | 0.092 |
| 119 | 1.6 | 150 | 0.256 | 0.105 | 3.320 | 810.0 | + 0.085 |
| 122 | 1.4 | 150 | 0.220 | 0.700 | 4.720 | 148.0 | 0.104 |
| 93 | 2.7 | 85 | — | — | no meas. activity | | |
| 97 | 2.1 | 50 | — | — | no meas. activity | | |
| 98 | 2.3 | 50 | — | — | no meas. activity | | |
| 99 | 1.8 | 110 | — | — | no meas. activity | | |
| 121 | 1.5 | 65 | — | — | no meas. activity | | |
| 124 | 2.4 | 90 | — | — | no meas. activity | | |
| 131 | 3.1 | 110 | — | — | no meas. activity | | |
| 132 | 2.7 | 100 | — | — | no meas. activity | | |
| 134 | 1.9 | 105 | — | — | no meas. activity | | |
| 136 | 1.4 | 85 | — | — | no meas. activity | | |
| 151 | 1.2 | 75 | — | — | no meas. activity | | |
| 161 | 2.9 | 60 | — | — | no meas. activity | | |

very difficult to interpret. SAUER (1954) described AChE containing cells in the spinal ganglion of the dog, using the same histochemical method.

The results of the present investigation, in analogy with those obtained by a histochemical technique on isolated spinal ganglion cells (GIACOBINI 1956), demonstrate that most of these cells (about 70 %) have a measurable ChE activity in their cell bodies and neurites, and that only a part of them show a low non spec. ChE activity together with AChE activity. Another group of cells shows activity, that was below the limit of detection, namely $10^{-6}$ $\mu$l $CO_2$/hour.

The ChE activity varies widely from cell to cell: in Table I a and 1 b *e. g.* it can be seen that the activity per unit of volume may be more than one hundred times higher in some cells than in others.

The higher ChE activity can by no means be attributed to the

**Table III.**

*Cholinesterase activity of single nerve cells from spinal ganglia of rat.*

| Diver no. | Cell body comp. diam. in $\mu$ | ChE activity/ unit area $\mu$l $CO_2/\mu^2$/h. (A) ($\times 10^{-3}$) | ChE activity/ unit volume $\mu$l $CO_2/\mu^3$/h. (B) ($\times 10^{-6}$) | Diver no. | Axon diam. | ChE activity/ unit area $\mu$l $CO_2/\mu^2$/h. (a) ($\times 10^{-7}$) | ChE activity/ unit volume $\mu$l $CO_2/\mu^3$/h. (b) ($\times 10^{-6}$) | Axon activity / Cell body activity | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | b/B | a/A |
| 65 | 34 | no meas. activity | | 99 | 1.8 | no meas. activity | | — | — |
| 103 | 36 | 10.240 | 172.0 | 100 | 2.0 | 7.060 | 1507 | 8.700 | 0.692 |
| 62 | 40 | no meas. activity | | 97 | 2.1 | no meas. activity | | — | — |
| 53 | 42 | 5.280 | 50.40 | 94 | 2.0 | 5.560 | 1077 | 21.20 | 1.050 |
| 101 | 45 | 0.446 | 5.880 | 109 | 2.4 | 1.432 | 239 | 40.06 | 3.200 |
| 70 | 45 | no meas. activity | | 98 | 2.3 | no meas. activity | | — | — |
| 42 | 52 | 0.536 | 6.160 | 91 | 2.8 | 1.240 | 181.5 | 29.30 | 2.310 |
| 79 | 58 | 0.100 | 1.030 | 93a | 3.2 | 0.670 | 84.60 | 82.20 | 6.700 |
| 64 | 58 | 0.105 | 1.080 | 87 | 3.2 | 1.100 | 142.5 | 131.5 | 10.40 |

greater dimensions of the cell, on the contrary, it is found that it is inversely correlated with the computed diameter, the surface or the volume of the cell. The inverse correlation of the activity and the cell body and axon surface is hardly compatible with the assumption that the enzyme should be localized in the cytoplasmic membrane and on the surface of the axon (NACHMANSOHN 1952).

The examination of the data shows that in the cells having ChE two different groups are present having high and low activity, and that the activity values plotted against the diameter, the surface or the volume fall along two separate curves of hyperbolic form. (See Fig. 4 A, B, C.)

The division into two distinguished groups of cells and the characteristic form of the activity curves of the cervical spinal ganglion cells may be of special interest. It is similar to the distribution of ChE activity found in the cervical anterior horn cells (GIACOBINI and HOLMSTEDT 1958) yet seems to be different from that found in the sympathetic ganglion cells (GIACOBINI 1957). This analogy can not be explained at present but could perhaps suggest the existence of a complete cholinergic pathway between the spinal cord and the periphery.

Comparing the activity per unit surface or volume of the cell body with its axon the latter is found to be several times higher in one case for example up to 131 times (unit of volume, Table III).

From the same relationship it becomes evident that smaller cells which show a higher activity in their cell bodies have a lower axon/body activity ratio. (See Table III.)

With regard to the absolute activity per unit volume, the spinal ganglion cells show about the same variation range found in the sympathetic ganglion cells (GIACOBINI 1957) but their absolute activity is about 10—20 times lower than that of the anterior horn cells (GIACOBINI and HOLMSTEDT 1958). In the present investigation no connection could be found between the two types of ganglion cells ("light" and "dark" cells) distinguished by morphologists (LENHOSSEK 1886, FLEMING 1895, COX 1898, CAJAL 1898—1906, HATAI 1901—1907, RANSON 1908—1912, BEAMS *et al.* 1952, HESS 1955) and their ChE activity.

The ChE activity determined with macrochemical methods on the whole anterior and posterior spinal roots of dog was calculated to be about 4—5 times lower in the latter (BURGEN and CHIPMAN 1951), nevertheless, WOLFGRAM (1954) reported approximately the same values for the dorsal and the ventral roots of cattle.

Several authors have demonstrated that acetylcholine is present in all types of peripheral nerves (BULLOCK *et al.* 1947, EULER 1947) but the ratio of the acetylcholine content of ventral/dorsal roots has been reported to vary between 300—180 (MACINTOSH 1941) and 80 (LOEWI and HELLAUER 1938) in the cat, and between 112 (LOEWI and HELLAUER 1938) and 52 (CHANG 1939) in the dog.

Even the synthesis of acetylcholine has been reported to be much lower in dorsal than in ventral roots (FELDBERG and MANN 1946, FELDBERG and VOGT 1948, WOLFGRAM 1954, ZETLER and SCHLOSSER 1955, HEBB and SILVER 1956). However, in comparing these apparent discrepancies, the different species used by various workers should be taken into consideration.

The evidence that part of the cells of the spinal ganglia, estimated by means of histochemical and microchemical methods to be approximately 10—15 % of the whole cell population, shows high AChE activity explains the presence of the low AChE activity found in the macrochemical determination on whole ganglia or in dorsal roots. If we assume that the small quantity of ACh and choline acetylase demonstrated by means of macrochemical methods are localized in the same neurones, this suggests the existence of a special type of sensory neurone of cholinergic nature.

On the other hand pharmacological evidence has been presented speaking in favour of the sensitivity to acetylcholine of practically all kind of sensory receptors and a release of acetylcholine has been demonstrated after stimulation of sensory nerves. However, at the present a definitive demonstration that a specific sensory stimulus may be related to a releasing mechanism of acetylcholine has not yet been produced.

## Summary.

1. The cholinesterase content of neurones belonging to the spinal ganglion cells of the rat has been investigated microchemically by means of a Cartesian diver technique.

The highest sensitivity reached by the method employed was $10^{-6}$ $\mu$l $CO_2$/hour with an accuracy of about 5 %.

2. Most of the cells examined showed a measurable ChE activity in their cell bodies and neurites, a part of these cells showed together with AChE activity a low non spec. ChE activity. An additional group of cells did not show any measurable activity.

3. It was found that the ChE activity varies significantly from cell to cell and the statistical analysis of the data showed that two different groups of cells are present; one group having relatively higher activity than the other. When the activity values are plotted against the diameter, the surface and the volume, they fall along two separate curves of hyperbolic form.

4. No direct correlation was found between either the cell diameter, the cell surface or the cell volume and the ChE activity.

5. Comparing the activity per unit of surface or of volume of the cell body with its axon, the latter was found to be several times higher.

6. With regard to the absolute activity per unit of volume, the spinal ganglion cells showed about the same variation found in the sympathetic ganglion cells (GIACOBINI 1957) but their absolute activity was about 10—20 times lower than that of the anterior horn cells (GIACOBINI and HOLMSTEDT 1958).

7. The results are discussed in the light of morphological and physiological data.

This work has been supported by grants from Karolinska Institutet.

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Acta physiol. scand. 1959. 45. 255—270.

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# Mechanism of the Release by Compound 48/80 of Histamine and of a Lipid-Soluble Smooth Muscle Stimulating Principle ("SRS").

By

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Received 3 November 1958.

**Abstract.**

CHAKRAVARTY, N., B. HÖGBERG and B. UVNÄS. — Mechanism of the release by compound 48/80 of histamine and of a lipid-soluble smooth muscle stimulating principle ("SRS"). *Acta physiol. scand.* 1959. *45*. 255—270. — Compound 48/80 releases histamine and a lipid-soluble smooth muscle contracting substance ("SRS") in the perfused cat paw. The time-course and dose-response curves of histamine and "SRS" release were strikingly parallel. Nonspecific enzyme inhibitors such as polysaccharides and "polysalicylic acid", and SH-blocking agents — such as allicin and iodoacetate — blocked the release of both histamine and "SRS". Heating of the paws to 45° C for 15 min had the same blocking effect. The observations support the hypothesis that compound 48/80 releases histamine in the cat paw by activating an enzymatic mechanism and that "SRS" is a product of the same or a similar mechanism.

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In sensitized tissue antigen-antibody reaction leads to the release of histamine and of another smooth muscle stimulating principle (KELLAWAY and TRETHEWIE 1940). Due to the slow course of the muscle contraction produced by this principle it is usually termed "slow reacting substance" ("SRS"). A principle causing a slow contraction of the guinea pig ileum has also been found in cat and dog plasma after intravenous administration of compound 48/80 (PATON 1951). The chemical nature of these principles is unknown.

Recently HÖGBERG *et al.* (1957), HÖGBERG and UVNÄS (1957, 1958), UVNÄS (1958) presented the theory that compound 48/80 causes degranulation of rat mast cells by activating a lytic enzyme attached to the mast cell membrane. The theory was supported by observations on the influence of temperature, pH, ionic milieu and enzyme inhibitors on the action of compound 48/80 (HÖGBERG and UVNÄS, 1959).

According to the experimental evidence accumulated in our laboratories, the perfused cat paw is rather sensitive to histamine liberators such as compound 48/80 (HÖGBERG, TUFVESSON and UVNÄS 1956). These liberators cause the release in the paw of considerable amounts of histamine and also of another smooth muscle stimulating principle ("SRS").

The only shown effect of small doses of compound 48/80 is its degranulating action on mast cells with the consequent release of histamine and in some species also of other biologically active substances stated to occur in the mast cells, such as 5-hydroxy-tryptamine and heparin. If compound 48/80 disrupted the mast cells by activating a lytic enzyme system in the cell membrane, hydrolysis products from the membrane might occur concomitantly with the histamine.

Lipid-soluble acids, such as acetalphosphatidic acid, and other acidic phospholipids claimed to occur in intestinal villi (VOGT 1957), unsaturated fatty acids (LASER 1950, GABR 1956) and other active principles of lipid-soluble acidic nature, such as prostaglandin and vesiglandin (EULER 1935, 1936) etc., produce slow contractions of smooth muscles.

The present observations on the effect of compound 48/80 in the perfused cat paw are consistent with the idea that the "SRS" occurring in our experiments is a biologically active lipid residue — possibly originating from the mast cells — although not identical with any of the smooth muscle contracting agents mentioned above.

## Methods and Materials.

*The cat paw technique.*

Isolated cat paws were perfused according to the technique described by HÖGBERG, TUFVESSON and UVNÄS (1956). A pair of paws was mounted on a polythene disc and perfused via the tibial artery with Tyrode solution at a temperature of about 30° C. All injections were made into the perfusion fluid just above its entry into the artery.

The paw of the cat is rather sensitive to compound 48/80, 1—10 $\mu$g of this compound yielding considerable amounts of histamine. The responses of the paws however vary considerably from cat to cat, but taken in pairs from the same animal they show a rather good quantitative correlation as to the release of both histamine and "SRS". In the present experiments, therefore, the observations were made on pairs of paws, one paw of the pair serving as the control.

The perfusion fluid was collected in tubes placed in iced water and then kept frozen until used for testing on the guinea pig ileum.

*Assay methods.*

Histamine was assayed on atropinized guinea pig ileum. The values are expressed as histamine base.

"SRS" was assayed on guinea pig ileum in the presence of atropine and an antihistamine (mepyramine). The standard used was prepared from sensitized guinea pig lungs, the "SRS" values being expressed in arbitrary units referring to the standard. The details about the standard and the method of assay will be described in a separate communication from this laboratory (CHAKRAVARTY, 1959).

Both histamine and "SRS" were usually tested directly in the perfusate. As will be described in the text, in control experiments "SRS" was extracted with alcohol and with ether before testing.

We are aware that under certain conditions compound 48/80 may influence the response of the guinea pig gut to histamine and "SRS" (PATON 1951), but in our experience the small concentrations of compound 48/80 that could be present in our perfusates had no appreciable effect on the sensitivity of the guinea pig ileum to histamine or "SRS" (CHAKRAVARTY, to be published).

*Compound 48/80.*[1]

The condensation product of p-methoxyphenethylmethylamine with formaldehyde was prepared according to the method described by BALTZLY *et al.* (1949).

*Pk 11.*[2]

Pk 11 is the code name of a polymer of salicylic acid.

[1] We are indebted to Dr. H. FEX for preparing compound 48/80.
[2] O. FERNÖ, B. HÖGBERG, and B. UVNÄS, to be published.

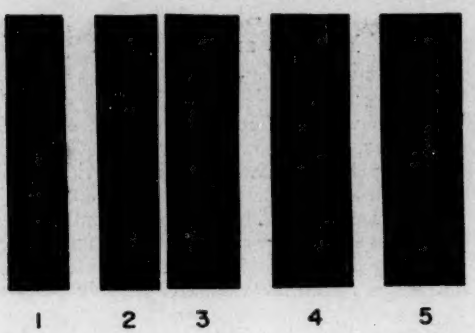


Fig. 1. Appearance of smooth muscle contracting principle ("SRS") in cat paw perfusate following administration of compound 48/80.

1. Original perfusate.
2 and 3. Two doses of alcohol extract.
4. Ether extract.
5. Methyl-ethyl-ketone extract.

Atropine $1.5 \cdot 10^{-6}$ M and mepyramine $5 \cdot 10^{-7}$ M added to the test bath.

*Hip seed polysaccharides.*

Hip seed polysaccharides were prepared from air dried ripe hips as described by HÖGBERG *et al.* (1957).

*Allicin.*

Allicin was extracted from garlic according to the technique described by CAVALLITO and BAILEY (1944).

## Results.

*Properties of "SRS".*

The contraction of the guinea pig ileum produced by "SRS" developed slowly. It started after a latency of about 5—30 sec, and rose to a peak in one to three min. The subsequent relaxation was slow in spite of repeated washing. "SRS" in freeze-dried material can be purified by extraction with 80 per cent alcohol. After adding 1—2 ml of N HCl to the dried residue it can be reextracted by organic solvents like ether, ethyl acetate, methyl-ethyl-ketone etc. By reextraction with alkaline water it can be recovered for testing on the guinea pig ileum. Fig. 1 shows the

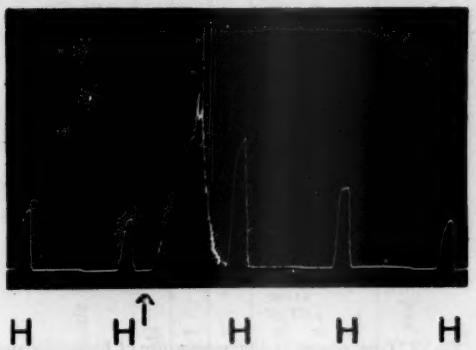


Fig. 2. Sensitization of guinea pig ileum to histamine by "SRS" (ether-extracted).

Atropine $1.5 \cdot 10^{-6}$ M and mepyramine $7.5 \cdot 10^{-6}$ M added to the test bath. H = histamine 0.9 $\mu$g added at intervals of 3—3.5 minutes. At the arrow "SRS" was added.

contractions caused by the active principle in the original perfusate and in extracts obtained as described above. It will be noticed that the nature of the contractions was not altered by the extraction procedures.

The slow reacting substance released in anaphylaxis has been shown to sensitize guinea pig ileum to histamine (BROCKLEHURST 1956). A similar sensitization to histamine response was observed with the "SRS" released by compound 48/80. Fig. 2 shows such an effect. The response to histamine was markedly potentiated by a small amount (0.06 ml) of a purified extract of "SRS".

A surprisingly good quantitative correlation was observed between the release of histamine and "SRS", at least when moderate doses of compound 48/80 were used. Fig. 3 shows the time course of the release of the two principles after 25 $\mu$g compound 48/80. It may be seen that the two curves run rather parallel during the 60 min of observation. Initially there is a slight time difference between the two curves. The reason for the delay of the "SRS" curve is obscure. It might possibly be due to different diffusion rates of the two active principles, the "SRS" molecule possibly being a rather large one (vide infra). Table I shows the appearance of histamine and "SRS" per 5 and 10 min periods during the hour following the administration of compound 48/80. In all experiments the appearance of the two prin-

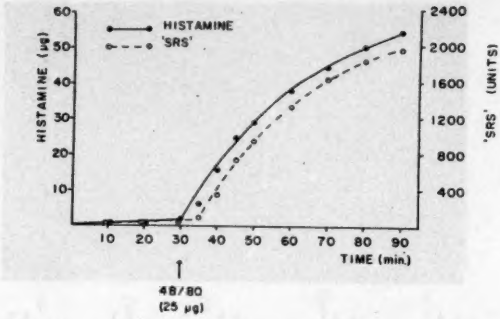


Fig. 3. Time course of the appearance of histamine and "SRS" in cat paw perfusate after administration of compound 48/80. Samples pooled from two paws.

ciples shows a good quantitative correlation. This is also the case in the experiment presented in Fig. 4, where — using the four paws of the same cat — the effect of various doses of compound 48/80 was plotted against the release of histamine and "SRS" during a 60 min period.

Preincubation of rat mast cells at temperatures around 45° C was reported by HÖGBERG and UVNÄS (1957) to render the cells insensitive to compound 48/80. The influence of heating a cat paw to 45° C for 15 min before perfusion with Tyrode solution at

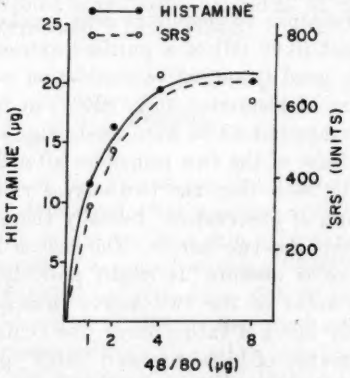


Fig. 4. Dose-response curves for histamine and "SRS" release caused by various doses of compound 48/80.

**Table I.**

*Time course of the appearance of histamine and "SRS" in cat paw perfusate following injection of compound 48/80.*

| Exp. no. | 5 min periods | | | | | | | | | | 10 min periods | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | |
| | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" |
| 1 | 1.4 | 0 | 4.7 | 38 | 6.3 | 102 | 5.2 | 95 | | | 11.2 | 173 | 9.9 | 142 | 8.0 | 113 | 6.5 | 88 |
| 2 | 2.2 | 18 | 4.8 | 140 | 4.4 | 196 | 2.2 | 106 | | | 4.5 | 319 | 3.3 | 161 | 2.7 | 99 | 2.0 | 59 |
| 3 | 0.7 | 1 | 0.7 | 2 | 0.8 | 6 | 2.0 | 24 | 2.4 | 38 | 4.5 | 102 | 5.7 | 186 | 4.7 | 109 | 3.9 | 97 |

H = histamine in $\mu$g. "SRS" expressed in units. Total collection period: 60 min for exp. 1 and 2, 65 min for exp. 3.

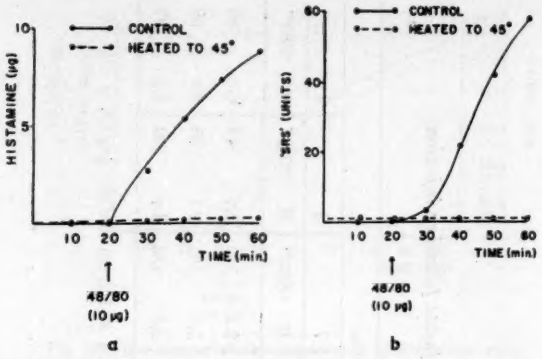


Fig. 5. The blocking effect of heating the paw to 45° C on the appearance of histamine (left) and "SRS" (right) in the perfusate.

the usual temperature (30° C) is seen in Fig. 5 a and b. In the control paw — the one of a pair — 10 µg compound 48/80 released 9 µg histamine and 60 units of "SRS" in 40 min. From the heated paw no histamine and no "SRS" appeared.

Various enzyme inhibitors were shown to block the degranulating action of compound 48/80 on rat mast cells (HÖGBERG and UVNÄS 1957, 1958). A few of these inhibitors were investigated

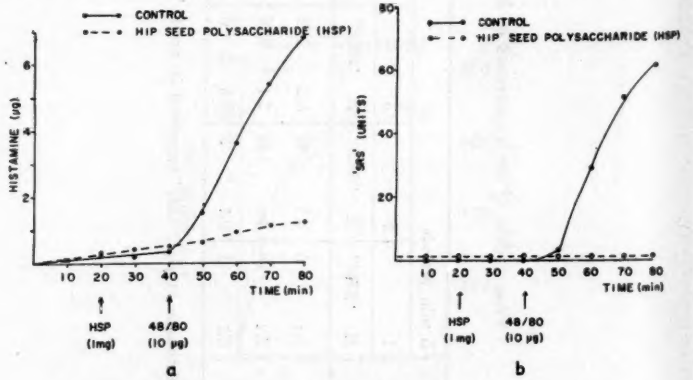


Fig. 6. The blocking action of hip seed polysaccharides on the appearance of histamine (left) and "SRS" (right) in the perfused cat paw after administration of compound 48/80.

**Table II.**

*Blocking action of enzyme inhibitors on the appearance of histamine and "SRS" in the perfused cat paw after administration of compound 48/80 10 μg.*

| Exp. no. | | 10 min periods | | | | | | | | Total | | % inhib. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 2 | | 3 | | 4 | | | | | |
| | | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" |
| 1 | Control | 2.9 | 12 | 8.5 | 51 | 3.1 | 19 | 5.4 | 22 | 19.9 | 104 | | |
| | Pk 11 (1 mg 10 min before inj. of 48/80) | 0.1 | 0 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0 | 99 | 100 |
| 2 | Control | 1.0 | 14 | 2.3 | 40 | 3.5 | 95 | 3.5 | 83 | 10.3 | 232 | | |
| | Pk 11 (1 mg 30 min before inj. of 48/80) | 0.1 | 0 | 0.1 | 0 | 0.1 | 0 | 0.1 | 0 | 0.4 | 0 | 96 | 100 |
| 3 | Control | 2.7 | 7 | 9.0 | 24 | 9.2 | 42 | 9.1 | 34 | 30.0 | 107 | | |
| | Allicin (200 μg 10 min before inj. of 48/80) | 2.8 | 4 | 2.5 | 6 | 3.2 | 7 | 2.5 | 4 | 11.0 | 21 | 63 | 80 |
| 4 | Control | 1.2 | 3 | 2.1 | 26 | 1.8 | 22 | 1.4 | 10 | 6.5 | 61 | | |
| | Hip seed polysacch. (1 mg 20 min before inj. of 48/80) | 0.1 | 0 | 0.3 | 0 | 0.2 | 0 | 0.1 | 0 | 0.7 | 0 | 89 | 100 |
| 5[1] | Control | 6.5 | 36 | 4.0 | 16 | 2.6 | 11 | 1.6 | 10 | 14.7 | 73 | | |
| | Mono-iodo-acetate ($10^{-3}$ M in perfusion fluid) | 1.0 | 0 | 0.7 | 0 | 0.6 | 0 | 0.5 | 0 | 2.9 | 0 | 80 | 100 |

[1] In exp. 5 15 min periods were used, in all others 10 min periods.

H = histamine in μg. "SRS" expressed in units.

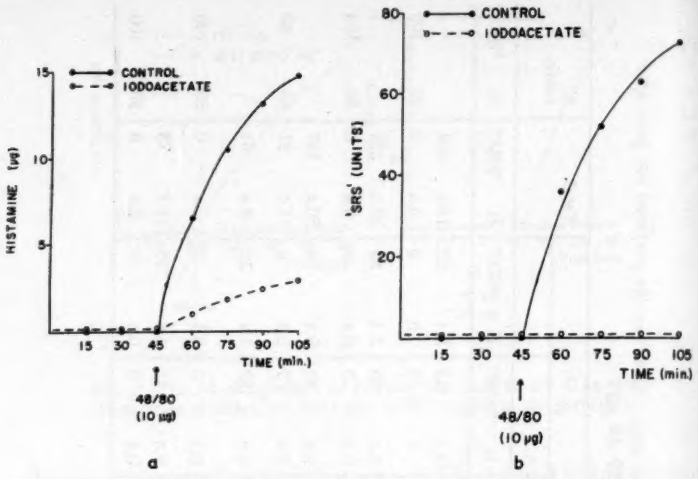


Fig. 7. The blocking action of mono-iodo-acetate on the appearance of histamine (left) and "SRS" (right) in the perfused cat paw after administration of compound 48/80.

with respect to their inhibitory action on the effect of compound 48/80 in the cat paw.

A polysaccharide fraction prepared from hip seeds was observed to block the release of histamine from the perfused cat paw and the degranulation of rat mast cells produced by compound 48/80 (HÖGBERG *et al.* 1957; HÖGBERG and UVNÄS 1957, 1958). The fact that these polysaccharides also prevent the appearance of "SRS" is evident from Fig. 6 a and b. In the experiment shown in this figure 1 mg of hip seed polysaccharides was injected into the tibial artery. The perfusion with Tyrode solution was then continued for 20 min before compound 48/80 was administered. Still the action of this compound was almost completely blocked. As illustrated in Table II, the synthetic polymer of salicylic acid, Pk 11, exerts a similar inhbitory action on the appearance of the two substances. As high molecular weight polymeric anions are known to be rather nonspecific enzyme inhibitors, these observations do not give any information as to the intimate nature of the enzymatic mechanism. Since SH-blocking substances were observed to inhibit the degranulating action of compound 48/80 on rat mast cells, two

**Table III.**

*Appearance of histamine and "SRS" from cat's paw perfused with oxygenated and oxygen-free Tyrode solution.*

| Exp. no. | | 10 min periods after 48/80 injection | | | | | | | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 2 | | 3 | | 4 | | | |
| | | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" |
| 1 | $O_2$..... | 2.4 | 36 | 4.6 | 119 | 3.2 | 102 | 3.2 | 73 | 13.4 | 330 |
| | $N_2$..... | 4.0 | 42 | 4.4 | 106 | 3.9 | 82 | 2.8 | 91 | 15.1 | 321 |
| 2 | $O_2$..... | 2.1 | 10 | 3.5 | 74 | 3.9 | 75 | 2.8 | 36 | 12.3 | 195 |
| | $N_2$..... | 5.0 | 71 | 4.3 | 125 | 3.0 | 73 | 2.9 | 61 | 15.2 | 330 |

H = histamine in $\mu$g. "SRS" expressed in units.

In exp. 1 the paws were perfused with the respective Tyrode solution for 40 min, in exp. 2 for 60 min before the injection of compound 48/80 10 $\mu$g.

such inhibitors were tested on the cat paw. Fig. 7 a and b presents an experiment with mono-iodo-acetate. The paw was perfused with Tyrode solution containing $10^{-3}$ M iodo-acetate, and after 45 min perfusion 10 $\mu$g of compound 48/80 was injected. The blocking action of iodo-acetate on the appearance of both substances is obvious. Another SH-blocking agent, allicin (WILLS 1956) has a similar inhibitory effect. The observations on the effect of the enzyme inhibitors used is summarized in Table II.

It is known since the report by PARROT (1942) that histamine release in anaphylaxis requires oxygen. The release of histamine from sensitized guinea pig lung in vitro is annulled or greatly reduced if the tissue is incubated in oxygen-free atmosphere. We perfused the cat paw with nitrogen-saturated Tyrode solution, but, as seen in Table III, even when the paw was perfused with oxygen-free Tyrode solution for 40—60 min before the injection of compound 48/80, there was no reduction in the amounts of histamine and "SRS" released. In one experiment pieces of skin from the cat's paw were incubated for 30 min with compound 48/80 (10 $\mu$g/ml) at 37° in oxygenated and oxygen-free (nitrogen-saturated) Tyrode solution. No significant difference was noticed between the histamine release in the two samples. It amounted to 1.3 $\mu$g/g tissue in oxygenated and 1.6 $\mu$g/g tissue in oxygen-free incubation fluid.

If our theory about the enzymatic histamine release from the

**Table IV.**

*Histamine and "SRS" in alcohol extracts of cat skin after compound 48/80, and after freezing and thawing of the skin.*

| Exp. | | Perfusate | | Alcohol extr. skin | | Freezing + thawing of skin | | Alcohol extr. of skin after freezing + thawing | |
|---|---|---|---|---|---|---|---|---|---|
| | | H | "SRS" | H | "SRS" | H | "SRS" | H | "SRS" |
| A | Control .... | 0 | 0 | 17.2 | 0 | | | | |
| | 48/80 ..... | 11.3 | 68 | 14.4 | 220 | | | | |
| B | Control .... | 0 | 0 | 8.1 | 0 | 10.0 | 0 | 2.4 | 0 |
| | 48/80 ..... | 15.3 | 280 | 7.1 | 110 | 8.3 | 68* | 1.7 | 66 |

Note that histamine and "SRS" values in the first column (perfusate) indicates total release during 30 min after compound 48/80 25 μg. In the other columns values are expressed as μg and units per g of tissue.

H = histamine in μg. "SRS" expressed in units.

* The contraction was not quite typical with too short latency and slow relaxation.

mast cells is correct, and if "SRS" is formed as a result of the same or a similar enzymatic process, it follows that "SRS" should not appear when histamine is released in non-enzymatic ways such as mechanical destruction of the cell membrane. The following experiments were made (Table IV A). A pair of cat paws was perfused with Tyrode solution and compound 48/80 25 μg was administered to one paw. In the perfusate from this paw 11.3 μg histamine and 68 units "SRS" appeared within 30 min. From the control paw neither histamine nor "SRS" appeared. After 30 min perfusion the skin of the two paws was extracted with 80 per cent alcohol and the extracts tested for histamine and "SRS". There was a clear cut difference between the two paws. The alcohol extracts from both paws contained considerable amounts of histamine but "SRS" could be obtained only from the paw treated with compound 48/80. The amount of histamine and "SRS" in the latter was 14.4 μg/g and 220 units/g respectively, whereas in the control paw 17.2 μg/g histamine but no "SRS" was found.

The experiment recorded in Table IV B was partially performed in the same way as the previous one shown in Table IV A. A pair of paws was perfused with Tyrode solution and 25 μg

compound 48/80 injected to one of the paws. After 30 min perfusion about one half of the skin of each paw was extracted with 80 % alcohol. The other half was subjected to repeated freezing and thawing and thereafter extracted with 80 % alcohol. As in the experiment shown in Table IV A, histamine was found in the alcohol extracts from both paws but "SRS" appeared only in the paw treated with compound 48/80. Freezing and thawing is known to release most of the tissue histamine. In our experiments we found that this procedure alone resulted in the release of about 80 % of the total histamine content of the cat skin. In other words, freezing and thawing releases histamine more completely than compound 48/80 does. In spite of this fact no "SRS" could be detected in the Tyrode solution in which the skin was suspended during freezing and thawing, nor could it be found in alcohol extracts of this skin. On the other hand, when compound 48/80 was administered to the paw, or, in other words, when an enzymatic histamine release was induced, "SRS" appeared in the alcohol extract of the skin following freezing and thawing.

## Discussion.

As mentioned in the introduction experimental evidence has been presented to support the hypothesis that compound 48/80 degranulates rat mast cells by activating a lytic enzyme attached to the mast cell membrane (HÖGBERG and UVNÄS 1957, 1958, 1959, UVNÄS 1958). In the present experiments the histamine release in the perfused cat paw produced by the same compound was observed to be completely abolished by heating the paw to 45° C. The histamine release was also inhibited by such non-specific enzyme inhibitors as polysaccharides and "polymeric salicylic acid" as well as by sulfhydryl blocking agents such as allicin and iodo-acetate. We therefore postulate that compound 48/80 releases histamine by activating an enzymatic mechanism in the cat paw also.

The time-course and the dose-response curves of histamine and "SRS" after the injection of compound 48/80 run surprisingly parallel. When the release of histamine is abolished by heating the paw to 45° C or by enzyme inhibitors, "SRS" also disappears from the perfusion fluid. It is evident therefore that the release of the two factors is linked together in some way, and might be the outcome of the same or closely linked enzymatic processes.

Our knowledge about the chemistry of "SRS" is thus far fragmentary. As already mentioned its distribution between ether and water is pH-dependent. It is unstable at room temperature, more so at higher pH (7—9) than at lower pH (4—6). However, when boiled with alkali or acid, higher inactivation is obtained in acid than in alkaline medium. On a one-dimensional paper chromatogram (solvent: n-propanol-ammonia-water (60 : 30 : 10): paper: Whatman No. 1) it has an Rf value of about 0.6—0.7. The active spot on the chromatogram does not show any of the commonly used staining reactions, but after strong acid hydrolysis of "SRS" (6 N HCl; 108° C; 20 hours; in a sealed tube) a ninhydrin-positive reaction appeared. No convincing staining reaction for phosphate has thus far been obtained. The active spot gives a yellow staining reaction with bromothymol blue.

The few available chemical data about "SRS" do not allow any far reaching conclusions as to the chemical nature of "SRS", but they all are consistent with the conception that it is a lipid-soluble acid compound. Such a substance might be expected to occur as a split product if compound 48/80 degranulated the mast cells by activating a lytic enzymatic mechanism. The correlation between histamine and "SRS" released by compound 48/80 under different experimental conditions suggests that both the substances are of mast cell origin.

It has been shown in the present experiments that freezing and thawing and/or alcohol extraction releases histamine from the cat's skin. However no "SRS" could be detected. The appearance of "SRS" required treatment of the tissue with compound 48/80, *i. e.* it requires the enzymatic process started by compound 48/80 in our opinion.

Enzymatic processes are supposed to be involved in the histamine release occurring in anaphylaxis. Some enzyme inhibitors have been shown to prevent the histamine release from sensitized guinea pig lung in vitro (MONGAR and SCHILD 1957). It has been observed in our laboratories that enzyme inhibitors, including sulfhydryl-blocking agents, inhibit not only the degranulation of rat mast cells and the histamine release in the cat paw produced by compound 48/80 but also the release of histamine in sensitized guinea pig lung. Of special interest is the fact that, when the histamine release was abolished in the sensitized lung tissue, there was a concomitant blocking of the appearance of "SRS"

(CHAKRAVARTY and UVNÄS, to be published). The similarities with the findings in the cat paw are striking, and we feel inclined to believe that similar enzymatic processes are involved in histamine release in anaphylaxis and in release by compound 48/80. We have observed one difference, however. Anoxia abolishes the histamine release in anaphylaxis but has no inhibitory effect on the histamine release produced by compound 48/80 in the cat paw. Although the interpretation of this observation is difficult, it might mean that the two processes have a common final enzymatic pathway but that the anaphylactic reaction has an oxygen-dependent link in addition.

The mast cells of the guinea pig seem to differ considerably in their sensitivity to compound 48/80 from those of the rat and the cat. In concentrations below 1 : 1,000 it does not cause any appreciable histamine release in the guinea pig lung in vitro. This is in marked contrast to the very small doses, about 100—1,000 times smaller, which are needed to degranulate rat mast cells or release histamine from the cat paw. It seems to us that compound 48/80 in the guinea pig has a different mode of action than in the cat and the rat. Probably in the guinea pig histamine is released by compound 48/80 by a direct lytic action similar to the action of other surface-active agents such as octylamine, decylamine, lysolecithin etc. Such an interpretation is supported by the observation that enzyme inhibitors and anoxia were unable to block the histamine-releasing action of compound 48/80 in the guinea pig lung (SCHILD 1956).

The authors' thanks are due to fil. mag. MAIJA-LEENA VÄHÄTALO-LYSSARIDES for valuable help with the chemical data of our "SRS".

This investigation was supported by a grant from the HARALD and GRETA JEANSSON Foundation.

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Acta physiol. scand. 1959. 45. 271—277.

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# Effect of Disulfiram (Tetraethylthiuram Disulphide) on the Metabolism of Methanol in Rat Liver Homogenates.

By

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Received 6 November 1958.

**Abstract.**

KOIVUSALO, M. Effect of disulfiram (tetraethylthiuram disulphide) on the metabolism of methanol in rat liver homogenates. *Acta physiol. scand.* 1959. *45.* 271—277. — The effect of disulfiram on the accumulation of formaldehyde in liver homogenates incubated with methanol has been studied in rats after feeding them high doses of disulfiram (1.0—0.5 g per kg daily for 9 days). The catalase and xanthine oxidase activities of the livers have also been determined. The formation of formaldehyde has been determined photometrically. The accumulation of formaldehyde in the liver homogenates from disulfiram-treated rats was not increased, but slightly inhibited by almost lethal doses. It was not stimulated by ATP as in normal animals. The liver catalase activity was clearly lowered in the disulfiram-treated rats. The liver xanthine oxidase activity was also diminished in these rats but not constantly.

Since the original discovery of the sensitizing effect of disulfiram (antabuse, tetraethylthiuram disulphide) on ethanol by HALD, JACOBSEN and LARSEN in 1948, it has been widely used in the therapy of chronic alcoholism. Most of the investigations on its mode of action in the organism have dealt with its effects on the metabolism of ethanol and acetaldehyde. The administration of

disulfiram *in vivo* before or simultaneously with ethanol causes accumulation of acetaldehyde in the blood (HALD and JACOBSEN 1948, LARSEN 1948). Its general effects on the metabolism are largely unknown, although inhibition of the endogenous tissue respiration and of various enzymes by disulfiram *in vitro* has been described (EDWARDS 1949, KJELDGAARD 1949, RICHERT, VANDERLINDE and WESTERFELD 1950, GRAHAM 1951, NYGAARD and SUMNER 1952). Of the effects of disulfiram on the metabolism of methanol very little is known, although it has a considerable toxicological and theoretical interest. Disulfiram is known to increase the acute toxicity of methanol to mice (GILGER, POTTS and JOHNSON 1952) and to retard considerably the elimination of administered methanol from the blood of rabbits (KOIVUSALO 1956 a, b). Disulfiram added *in vitro* to guinea pig liver homogenates inhibited markedly the utilization of methanol (KOIVUSALO 1956 b). The poor solubility of disulfiram has rendered its use in the *in vitro* experiments difficult, its solubility in water being only about 40 $\mu$g per 10 ml according to KJELDGAARD (1949). This difficulty is partly overcome by using organs from disulfiram-treated animals.

In the present paper results are presented from experiments in which homogenates prepared from livers of rats treated with high doses of disulfiram have been incubated with methanol and the accumulation of formaldehyde has been determined. The catalase and xanthine oxidase contents of the livers of the rats treated with disulfiram have also been determined in view of the generally assumed close relationship of these enzymes with the oxidation of methanol in the animal organism (for a review see KOIVUSALO 1956 b).

## Methods.

Thirty white male rats of Wistar strain weighing 280—300 g were used as experimental animals. They were fed the usual stock diet of this Department.

Disulfiram (Tetraethylthiuram Disulphide, purum, Fluka AG, Chemische Fabrik Buchs/SG) was administered to the rats with food over a period of 9 days. The animals were divided in three groups, the first group was given 300 mg of disulfiram each per day, the second group 150 mg each per day and the third group received as controls the same diet without disulfiram.

Two to six rats from each group were killed on the third, sixth and ninth days of the experimental period. Of the livers 10 per cent homo-

genates were prepared with 0.1 M potassium phosphate buffer pH 7.4 as described earlier (KOIVUSALO 1956 b). The composition of the incubation mixture is described in the explanatory text of the table. The incubations were carried out in a water bath at 37° with constant shaking, using air as gas phase. Samples of 1 ml were taken from the incubation flasks before and after the incubation and transferred to centrifuge tubes containing 5 ml of cold trichloroacetic acid for deproteinization. The clear supernatant obtained after centrifugation was used for the determination of formaldehyde.

The determination of formaldehyde was made using a photometric method based on the chromotropic acid reaction (KOIVUSALO 1956 b). All the values in the tables are averages from duplicate analyses from two similar incubation flasks and corrected for the reagent and tissue blank values.

The catalase activity of the livers was determined by the perborate method of FEINSTEIN (1949). The assays were made using dilutions of liver tissue 1 : 1,000 and 1 : 500, the results given are averages from these determinations.

The xanthine oxidase activity was assayed using the manometric method of AXELROD and ELVEHJEM (1941) as modified by RICHERT, EDWARDS and WESTERFELD (1949), without addition of methylene blue. The values for xanthine oxidase activity are reported as $\mu$l of oxygen per 20 min per 283 mg of fresh liver.

## Results.

The used dosage of disulfiram was very high, but it has been found in earlier experiments (KOIVUSALO 1956 b) that with smaller amounts no effects could be obtained on the metabolism of methanol. After four days all rats in the present experiments had developed toxic symptoms, they were apathic and did not move in the cages. On the seventh day all the rats receiving 300 mg of disulfiram daily developed cramps and died.

When the results which are presented in Table I are examined, it is seen that the administration of disulfiram *in vivo* has no clear effect on the formation of formaldehyde from methanol in liver homogenates. When almost lethal doses have been administered as in rats no. 13—15 a small inhibitory effect is seen. However, the addition of adenosine triphosphate caused no or only a slight increase in the formation of formaldehyde in the homogenates from disulfiram-treated rats. In normal homogenates a marked increase in the formation of formaldehyde was consistently seen after the addition of adenosine triphosphate.

**Table I.**

*Formation of formaldehyde from methanol in liver homogenates from disulfiram-treated rats and the liver catalase and xanthine oxidase activities of these rats.*

5 ml of 10 per cent liver homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 800 μg of methanol. Total volume 7 ml. Incubated for 60 min at 37°. Gas phase air. Additions: 5 μmoles of ATP. Catalase activity according to FEINSTEIN (1949), xanthine oxidase activity as μl $O_2$ per 20 min per 283 mg fresh liver.

| Rat no. | Experimental period and diet | Formaldehyde formed μg | | Liver catalase activity | Liver xanthine oxidase activity |
|---|---|---|---|---|---|
| | | No additions | ATP added | | |
| 1 | 3 days, 300 mg of disulfiram per day | 36 | 63 | 0.48 | 32 |
| 2 | » | 41 | 64 | 0.41 | 24 |
| 3 | » | 59 | 73 | 0.54 | 36 |
| 16 | Control | 37 | 73 | 0.68 | 32 |
| 17 | Control | 44 | 95 | 0.65 | 35 |
| 4 | 6 days, 150 mg of disulfiram per day | 53 | 79 | 0.61 | 39 |
| 6 | » | 55 | 68 | 0.64 | 36 |
| 8 | » | 49 | 57 | 0.55 | 30 |
| 9 | » | 42 | 35 | 0.35 | 30 |
| 5 | 6 days, 300 mg of disulfiram per day | 60 | 51 | 0.58 | 29 |
| 7 | » | 66 | 73 | 0.58 | 27 |
| 18 | Control | 62 | 93 | 0.73 | 38 |
| 19 | Control | 41 | 78 | 0.70 | 30 |
| 10 | 9 days, 150 mg of disulfiram per day | 43 | 47 | 0.33 | 14 |
| 11 | » | 53 | 54 | 0.43 | 18 |
| 12 | » | 56 | 59 | 0.40 | 10 |
| 13 | » | 19 | 28 | 0.31 | 21 |
| 14 | » | 31 | 31 | 0.26 | 15 |
| 15 | » | 26 | 22 | 0.28 | 20 |
| 20 | Control | 47 | 76 | 0.78 | 29 |
| 21 | Control | 53 | 84 | 0.71 | 40 |
| 22 | Control | 37 | 102 | 0.73 | 37 |
| 23 | Control | 60 | 95 | 0.69 | 33 |

The liver catalase activity of the disulfiram-treated rats was clearly lower than that of the normal controls. The liver xanthine oxidase activity was lowered only in some disulfiram-treated rats, in the others it was within normal limits.

## Discussion.

The liver xanthine oxidase activity in the present experiments has been very resistant to the administration of disulfiram *in vivo*, when compared to the marked inhibition of liver xanthine oxidase activity reported to occur when disulfiram has been added *in vitro* (RICHERT, VANDERLINDE and WESTERFELD 1950). However, variable results were obtained also by the afore-mentioned authors when disulfiram was fed to rats and the xanthine oxidase activities of the livers were determined. The dosage used by these authors was only 1/3—1/10 of that used in the present study.

The liver catalase activity was markedly diminished after the feeding of disulfiram. Administration of 150 mg of disulfiram daily for 9 days caused a decrease of 60 per cent in the liver catalase activity. DALE (1953) has determined the catalase activity of human liver specimens obtained by needle biopsy and has found normal values in disulfiram-treated alcoholics. The dosage level of disulfiram in these cases was, however, much lower than in the present animal experiments. No reports has been found concerning the effect of disulfiram on the activity of pure catalase preparations.

The administration of disulfiram *in vivo* caused in the present experiments no increase in the accumulation of formaldehyde in rat liver homogenates incubated with methanol. A slight inhibitory effect was seen when almost lethal doses of disulfiram were administered and this effect was very probably due to a decreased formation of formaldehyde from methanol. Apparently the enzymes responsible for the utilization of formaldehyde are not very sensitive to disulfiram as is demonstrated also by the small inhibitory effect of added disulfiram on the utilization of formaldehyde in guinea pig liver homogenates (KOIVUSALO 1956 b).

The low liver catalase activity of the disulfiram-treated rats explains readily the inhibition in the formation of formaldehyde from methanol, if it is assumed that methanol is mainly oxidized by peroxidatically functioning catalase (KEILIN and HARTREE 1945). The purified horse liver alcohol dehydrogenase is not affected by disulfiram *in vitro* (KJELDGAARD 1949). The lack of the stimulatory effect of adenosine triphosphate on the formation of formaldehyde in the liver homogenates from disulfiram-treated rats, may also be attributed to the low liver catalase activity in

these rats, and is in accordance with the catalase theory of methanol oxidation. Xanthine oxidase can use also adenosine triphosphate as substrate and the formed hydrogen peroxide can be used by catalase for the oxidation of methanol. The effect of adenosine triphosphate in normal homogenates can thus be due to stimulation of the peroxidatically functioning catalase.

This investigation has been supported by a generous grant from the Foundation for the Research of Alcohol Problems.

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Acta physiol. scand. 1959. *45*. 278—282.

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# Acetylation of p-Aminobenzoic Acid in Various Animal Tissues in vitro.

By

MARTTI KOIVUSALO and TAPANI LUUKKAINEN.

Received 6 November 1958.

**Abstract.**

KOIVUSALO, M. and T. LUUKKAINEN, Acetylation of *p*-aminobenzoic acid in various animal tissues in vitro. *Acta physiol. scand.* 1959. *45*. 278—282. — The acetylation of *p*-aminobenzoic acid has been studied *in vitro* in rat heart, lung, kidney and liver preparations as well as in ox kidney. The method of BRATTON and MARSHALL (1939) has been used for the determination of diazotizable amines. The experiments were carried out with tissue preparations cut into small pieces with scissors. All of these tissues were able to convert *p*-aminobenzoic acid to non-diazotizable derivatives, which were assumed to be acetyl conjugates. This has not been shown earlier with ox kidney tissue or with the heart and lung of any animal.

The acetylation of *p*-aminobenzoic acid and sulphanilamide is generally assumed to take place mainly in the liver (HARRIS and KLEIN 1938, STEWART, ROURKE and ALLEN 1939, KINNUNEN 1946, KREBS, SLYKES and BARTLEY 1947, PHILLIPS and ANKER 1957). It has been shown in this laboratory that preparations of rat kidney are able to acetylate *p*-aminobenzoic acid (LUUKKAINEN 1958). In this paper are reported the results of experiments on the acetylation of *p*-aminobenzoic acid in some other tissues. The tissues tested were rat heart and lung and ox kidney.

## Methods.

The rats used were of Wistar strain. The animals were killed by decapitation, the organs were removed and immediately placed on ice. The ox kidneys were obtained from the local slaughterhouse. The kidneys were quickly removed without injury and were cleaned of fat and connective tissue, cut in pieces and kept on ice until used.

The experiments were carried out with tissue preparations cut with scissors in the incubation medium into small sections or "cuttings". The amount of tissue used was 2 g in most experiments. In some experiments only 1 g of tissue was used.

The composition of the incubation medium was as follows:

0.9 per cent sodium chloride, 200 ml
0.154 M potassium chloride, 8 ml
1.0 M sodium acetate, 0 or 8 ml
0.02 M neutralized *p*-aminobenzoic acid, 2—16 ml (to give the desired substrate concentration in 10 ml)
0.1 M potassium sodium phosphate buffer, pH 7.4, to make 400 ml.

Of this incubation medium 10 ml were used in each incubation flask. The flasks were gassed for 10 minutes with oxygen. Thereafter they were incubated in a temperature-controlled water bath at 37° for 4 hours with constant shaking. After incubation duplicate samples of 1 ml were taken from each flask and transferred to centrifuge tubes containing 4 ml of 5 per cent trichloroacetic acid. The clear supernatant obtained after centrifugation was used for the analyses.

The diazotizable amines were determined by the method of BRATTON and MARSHALL (1939) before and after hydrolysis. The difference in these determinations was assumed to represent the acetyl conjugate.

The experimental technique and the methods used have been described in further detail by LUUKKAINEN (1958).

## Results.

The results of typical series of experiments are presented in Table I and II and in Fig. 1.

In the experiments with the different rat tissues (Table I) it was found that in addition to liver and kidney tissues also lung and heart tissues had considerable acetylating activity.

Some experiments were also carried out using rat liver homogenates prepared with a blendor-type homogenizer. No acetylating activity was found in any of these experiments.

Because of the small size of the rat kidney it was difficult to obtain enough material for further studies in the acetylating

**Table I.**

*Acetylation of p-aminobenzoic acid (PAB) in different rat tissues.*

Incubated at 37° for 4 hours using oxygen as gas phase. The incubation medium, pM 7.4, contained 200 μmoles of acetate per 10 ml.

| Tissue | Amount of tissue g | PAB added μmoles | Acetyl-PAB formed μmoles |
|---|---|---|---|
| Liver | 2 | 2 | 1.20 |
| Liver | 2 | 2 | 1.17 |
| Lung | 2 | 1 | 0.61 |
| Lung | 2 | 1 | 0.71 |
| Heart | 2 | 2 | 0.68 |
| Heart | 2 | 2 | 0.32 |
| Kidney | 1 | 2 | 0.43 |
| Kidney | 1 | 2 | 0.32 |

**Table II.**

*Acetylation of p-aminobenzoic acid (PAB) in ox kidney.*

Experimental conditions as in Table I.

| Tissue | Amount of tissue g | PAB added μmoles | Acetyl-PAB formed μmoles |
|---|---|---|---|
| Cortex | 2 | 2 | 1.84 |
| Cortex | 2 | 2 | 1.84 |
| Cortex | 2 | 2 | 1.78 |
| Medulla | 2 | 2 | 0.65 |
| Medulla | 2 | 2 | 0.67 |
| Medulla | 2 | 2 | 0.68 |
| Cortex + medulla | 1 + 1 | 2 | 1.18 |
| Cortex + medulla | 1 + 1 | 2 | 1.16 |

system in kidney tissue. The acetylation of *p*-aminobenzoic acid was therefore studied also in ox kidney tissue (Table II). It was observed that there occurred a marked acetylation of *p*-aminobenzoic acid and that this activity was mainly localized in the cortex.

In the experiments presented in Fig. 1 the effects of the substrate concentration and of the omission of acetate from the incubation medium were studied in ox kidney tissue. It was observed that although an appreciable quantity of *p*-aminobenzoic acid was acetylated without added acetate, the addition of 200 μmoles of acetate stimulated the acetylation very markedly.

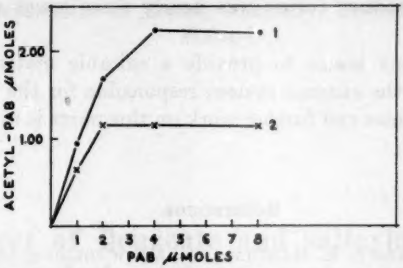


Fig. 1. Acetylation of *p*-aminobenzoic acid (PAB) in ox kidney cortex with and without added acetate.

2 g of kidney cortex in 10ml of incubation medium pH 7.4.

Curve 1. Standard incubation medium with 200 μmoles of acetate per 10 ml.
Curve 2. Incubation medium without acetate.

Ordinate: Amount of acetyl PAB formed during 4 hours, in μmoles.

Abscissa: Amount of PAB used as substrate, in μmoles.

Gas phase: oxygen. 37°. Each point represents an average of results from three similar incubation flasks.

## Discussion.

The present experiments demonstrate clearly that *p*-aminobenzoic acid is acetylated *in vitro* also in cuttings of rat lung and heart tissue. The acetylating activity in these tissues was rather high, being of the same order of magnitude as in the liver and kidney tissues. However, conclusions concerning the significance of these organs as to the site of acetylation of aromatic amines *in vivo* cannot be drawn merely on the basis of *in vitro* experiments. In the recently published experiments of LAUENER *et al.* (1957) a slight formation of acetylated *p*-aminosalicylic acid was reported to occur after incubation of rat lung tissue slices with *p*-aminosalicylic acid.

No reports have been found in the earlier literature concerning the acetylation of *p*-aminobenzoic acid in heart and lung tissue or of aromatic amines in ox tissues, which, however, offer a better opportunity for preparative work on a larger scale. The acetylating activity of ox kidney tissue which has been observed in the present experiments was very marked and comparable to that found in the kidney tissue of young male rats (LUUKKAINEN

1958). The ox kidney cortex was nearly three times so active as the medulla.

The ox kidney seems to provide a suitable material for the purification of the enzyme system responsible for the acetylation of aromatic amines and further work on this point is in progress in this laboratory.

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Acta physiol. scand. 1959. 45. 283—287.

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# Effect of Benzoate and Salicylate on the Acetylation of p-Aminobenzoic Acid in Ox Kidney in vitro.

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Received 6 November 1958.

**Abstract.**

KOIVUSALO, M. and T. LUUKKAINEN. Effect of benzoate and salicylate on the acetylation of *p*-aminobenzoic acid in ox kidney in vitro. *Acta physiol. scand.* scand. 1959. *45.* 283—287. — The effect of benzoate and salicylate on the acetylation of *p*-aminobenzoic acid *in vitro* has been studied in the ox kidney cortex. Both salicylate and benzoate inhibited the formation of acetyl-*p*-aminobenzoic acid when their concentration was $10^{-2}$ M or more.

Benzoate and salicylate have been found to increase the acetylation of aromatic amines *in vivo* (RIGGS and CHRISTENSEN 1951, KOIVUSALO *et al.* 1958). On the other hand, benzoate and salicylate are known to inhibit many enzymic reactions. The present paper deals with the effect of benzoate and salicylate on the acetylation of *p*-aminobenzoic acid *in vitro* in the ox kidney cortex.

## Methods.

The tissue preparation used was ox kidney cortex cut into small sections with scissors. The amount of tissue was 2 g in each experiment. The experimental technique and methods have been described in detail earlier (LUUKKAINEN 1958).

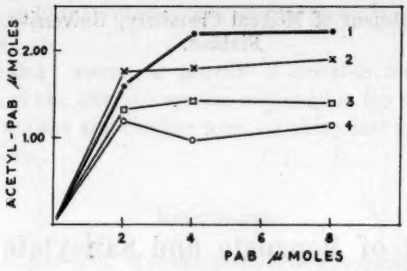


Fig. 1. Effect of benzoate on the acetylation of *p*-aminobenzoic acid (PAB) in ox kidney cortex.

2 g of kidney cortex tissue in 10 ml of incubation medium, containing 200 μmoles of acetate. Incubated at 37° for 4 hours, pH 7.4. Gas phase: oxygen.

Curve 1. No additions.
Curve 2. 20 μmoles of benzoate.
Curve 3. 100 μmoles of benzoate.
Curve 4. 200 μmoles of benzoate.

The desired amounts of sodium benzoate and sodium salicylate were added in 0.2 ml of 0.1 M phosphate buffer, pH 7.4, to the 10 ml incubation medium. In the control experiments 0.2 ml of the same buffer alone was added. The flasks were incubated for 4 hours at 37° with constant shaking, using oxygen as gas phase. The diazotizable amines were determined by the method of BRATTON and MARSHALL (1939).

## Results.

Fig. 1 shows the results of experiments performed to study the effect of benzoate on the acetylation of *p*-aminobenzoic acid using different amounts of substrate. When 20 μmoles of benzoate were added, an inhibitory action was seen if the amount of substrate was 4 μmoles or more. The inhibition was observed already with 2 μmoles of substrate when 100 or 200 μmoles of benzoate were used.

Similar experiments were also made with salicylate (Fig. 2). When 20 μmoles of salicylate were used no inhibition was found in the acetyaltion of 2—8 μmoles of *p*-aminobenzoic acid. When the amount of salicylata was increased to 100 μmoles a marked inhibition was observed, and 200 μmoles of salicylate caused a nearly complete inhibition of acetylation.

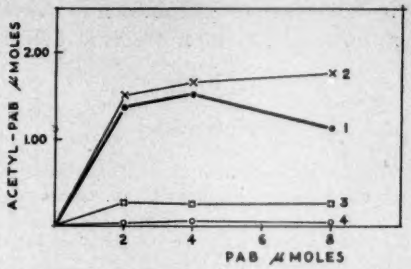


Fig. 2. Effect of salicylate on the acetylation of *p*-aminobenzoic acid (PAB) in ox kidney cortex.

Experimental conditions as in Fig. 1.

Curve 1. No additions.
Curve 2. 20 μmoles of salicylate.
Curve 3. 100 μmoles of salicylate.
Curve 4. 200 μmoles of salicylate.

## Discussion.

Benzoate and salicylate have been shown to have an inhibitory action on many metabolic functions. For example they inhibit tissue respiration (LUTWAK-MANN 1942, HERNER 1944), oxidative enzymes of the tricarboxylic acid cycle (KAPLAN, KENNEDY and DAVIS 1954) and incorporation of acetate into glycogen (SMITH 1958), and uncouple the oxidative phosphorylation from respiration in mitochondria (PENNIALL, KALNITSKY and ROUTH 1956, BRODY 1956, BOSUND 1957).

The present experiments demonstrate that both benzoate and salicylate inhibit the acetylation of *p*-aminobenzoic acid in the ox kidney cortex. The inhibitory effect of salicylate was greater than that of benzoate as has been observed also in other systems (*e. g.* HERNER 1944, BOSUND 1947).

The acetylation of aromatic amines requires energy rich phosphate (LIPMANN 1945), and thus the uncoupling of oxidative phosphorylation from respiration by benzoate and salicylate (PENNIALL *et al.* 1956, BRODY 1956, BOSUND 1957) may explain the inhibition found in the acetylation of *p*-aminobenzoic acid.

The discrepancy between the results obtained *in vivo* and *in vitro* experiments is probably due to a change in the excretion rate of *p*-aminobenzoic acid *in vivo* caused by benzoate and salicylate.

A decrease in the excretion of *p*-aminobenzoic acid after the administration of benzoate has been observed to occur in rats (LUUKKAINEN 1958).

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